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NASA TECHNICAL TRANSLATION NASA TT F-14,858

THE CAROTENOIDS OF SOME BASIDIOMYCETES: A CHEMIOTAXIONOMIC SURVEY

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Translation of: "Les carotenoides des basidiomycetes: Survol chimiotaxinomique", (Theses), University of Lyon, France, July 1968, 84+ pages.

(NASA-TT-F-14858) THE CAROTENOIDS OF SOME BASIDIOMYCETES: A CHEMIOTAXIONOMIC SURVEY (Linguistic Systems, Inc., Cambridge, Mass.) 103 p HC $7.25 CSCL 06A G3/04

N73-20117

Unclas 66671

NATIONAL AERONAUTICS AND SPACE ADMINISTRATION
WASHINGTON, D.C. 20546 APRIL 1973

FOREWORD

Before undertaking my description of this work, it is a pleasant duty to express my profound gratitude to all those who permitted its accomplishment.

To Professor R. Kühner and Professor J. Boidin I owe my first guidance in the path of scientific research: their teaching of Plant Physiology and Biochemistry awakened in me the taste for this study, fulfilled when they welcomed me into their Laboratory. Thus it is for me a great honor and pleasure to see them today on the Examining Board of my thesis; I would hereby like to express to them my respectful gratitude.

I am anxious to express every gratitude and my affection for Professor Ph. Lebreton, who welcomed me warmly into his Department and who has given me untiring advice and encouragement.

To Doctor H. Thommen (Ets. Hoffman-La Roche, Basel) and Doctor K. Egger, Privat-Dozent at the Botanical Institute of the University of Heidelberg, my appreciation for the fruitful periods of work they were kind enough to permit me in their laboratories.

My warmest thanks to Mr. N. Arpin who was my brotherly mentor in the field of carotenoids, and Miss M. P. Bouchez for her friendly and valuable collaboration. Nor do I forget all my workfellows in the Plant Chemistry and Plant Physiology Department, whose friendship greatly facilitated these researches. I thank them for the true privilege of belonging to their team.

Finally, my thanks to all the members of the Plant Biology Department, whose kind cooperation and competence assisted me in this work.

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CHAPTER I

/1*

FUNGUS CAROTENOIDS: GENERAL

Throughout mycological history, the extraordinarily rich and varied range of colors of fungi has initiated many a branch of this science and has been used by taxonomists to define and classify the species. Such regroupings, carried out on the basis of common colorations, implies common origins and assumes that one and the same chemical compound is involved; thus the Fries classification of the Agaricales based on the color of the spurry, was already chemotaxonomic.

The elucidation throughout the last decade of the mechanisms of biological syntheses and the concretization of hereditary characteristics allows us to confirm the following intuitive inference: the presence of a pigment - as of any other chemical compound - is only the visible manifestation of the plant possessing a certain enzyme system, namely, to begin with, certain "nucleoprotein information." Indeed, we have a chain of causation: DNA - messenger RNA - protein (especially enzyme) - chemical product or substrate. Pigment analysis is thus only a new manner of "apprehending the genome through the phenome", more surprising at first view but also classical in its foundation and probably more direct than traditional systematic criteria.

Just as the various fossils in a stratum are not all of equal interest to the geologist, the various compounds synthesized by a living being are of extremely varying value in the eyes of the taxonomist. Possession of a certain molecule or biogenetic family is usually more significant, or involves a narrower range of taxons, the less frequent it is. This obviously excludes from the first rank the elements in metabolic chains essential to life: glucose, and ATP for example, encountered in all living creatures. As a parallel to the above, the position of a biochemical family on a path more or less important for the economy of the organism will leave it only a more or less narrow latitude of variation from one system group to another, as they may be characterized. The products of biosynthetic activity can thus be roughly classified into:

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*Numbers in righthand margin indicate pagination of foreign text.

- functional molecules, being the metabolic framework of the living material;

- reserve molecules, particular end-points of anabolism but also points of departure differing from energy catabolism (starch and various analogous polyosides);

- structural molecules (cellulose, chitin, lignin, etc.) accumulated "with no hope of return";

- excretion or waste molecules which, once formed, seem to have no part to play whatever; carotenoid pigments, at least in heterotrophic organisms come under this category.

Throughout this classification, the route is as follows: from the most "active" to the most "inert", from the most universal to the most specific, the latter being richest in taxonomic significance. Thus if, because of their apparentness to the observer, the pigments were among the first compounds studied more or less implicitly from this viewpoint, this choice has, in particular for the plant group which concerns us here, proved to have a rational basis.

But the color, a macroscopic and anthropomorphic characteristic, is a very fragile element. Like any other morphological datum, it can be the subject of convergence phenomena: two colorings very close to one another, even identical, can be made of two totally different pigments which, far from indicating a relationship, show a divergency in chemical orientation. However, here, the value of superficial resemblance is easily measured by the spectrophotometer: thus Clitocybe venustissima and Hygrophoropsis aurantiaca have very close orangy colors which, a priori, could warrant the two species being linked together; but although the color of the first is carotenoid, the coloring matter of the second is quite different [5]. However, before /3
physico-chemical techniques of analysis reached the mycology laboratories, the characteristics of solubility and cytological location of pigments, in direct relationship with their structure, had already provided valuable information of great advantage to taxonomers [98].

Pastac [123] published a review of the various fungus pigments from the chemical viewpoint (quinones, in particular anthraquinones; polyeric colorants (of which carotenoid is one), and heterocyclic colorants, while Heim [83] sought to emphasize their systematic role. Although fungus carotenoids were not considered in Hegnauer's book [77], they were described, from

different points of view, by Goodwin [60], Haxo [76], and, more recently, by Valdon [153]. After showing the structures and biogenetic relationships of carotenoids, we will set up a table of what is presently known of their distribution in fungi. This will permit us to show the characteristics and particularities of this carotenogenesis, then their value to the taxonomist and in some cases phylogenetic conclusions may be drawn.

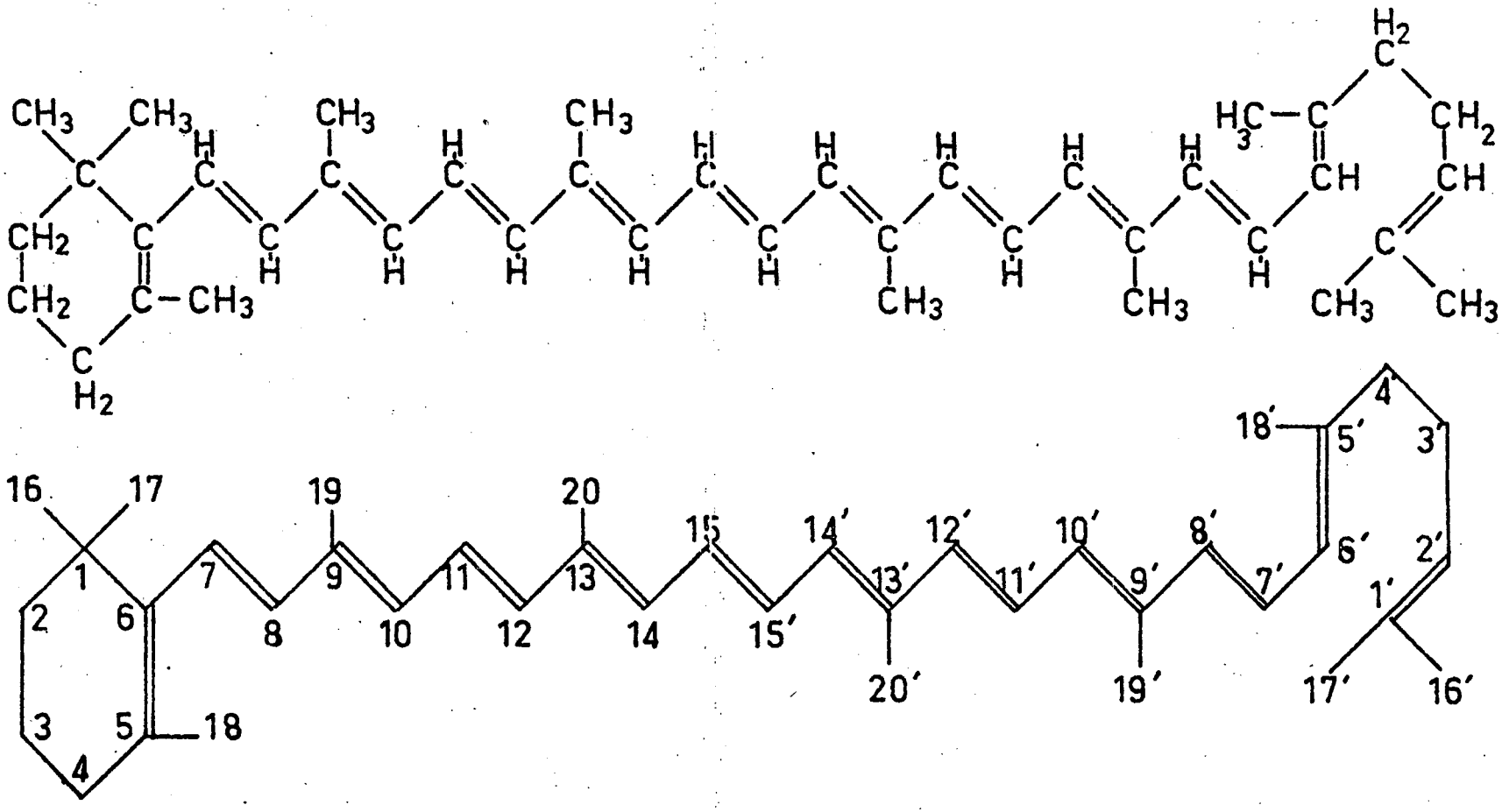
1. STRUCTURE AND BIOSYNTHETIC SEQUENCES OF CAROTENOIDS

The liposoluble and unsaponifiable pigments, yellow, orangy or red in color, called "carotenoids" by Tswett in 1911 (previously known as lipochromes) are compounds with forty carbon atoms, with a system of conjugated double bonds which originates the coloring; "on paper" they can all be connected by a series of reactions: cyclization, dehydrogenation, hydrogenation, hydration, oxygenation. One of them, γ-carotene, will illustrate their structure.

Two groups of compounds, although they do not exactly fit /4
this definition, must be grouped here. These are, first, colorless products (since they are only somewhat unsaturated): phytoene (I) and phytofluene (II) and, second, apo-carotenoids which possess less than forty carbons in the molecule and result from the oxidative degradation or scission of carotenoids properly speaking; neurosporoxanthin (XXVI) is an example.

From the essentially practical viewpoint, the carotenoids can be divided into carotenes, which are hydrocarbons, and xanthophylls, whose molecule also contains oxygen. Another classification separates the acyclic, monocyclic, and bicyclic carotenoids which are respectively represented by neurosporene (VI), γ-carotene (XVI) and β-carotene (XXVII); such a distinction is all the more natural since, as we shall see, it corresponds to the broad directions in which biosynthesis can be involved in these pigments, while the passage from carotenes to xanthophylls is no more than one and the same stage passed independently on different pathways.

The diagram below of carotenoid biosynthesis as generally accepted today [63, 108, 28] or as it may be postulated according to the structure of pigments studied very recently [1, 43, 21, 9, 10, 51] shows that the hydrocarbon skeleton first undergoes progressive oxidation by dehydrogenation. At least for the major pigments, it is only when this desaturation is fairly strong (at neurosporene IV) that the two great diversification processes of this family of pigments come into play: terminal cyclization


CH3
CH2
H2C
H3C
16
17
19
20
18
18′
20′
19′
17′
16′

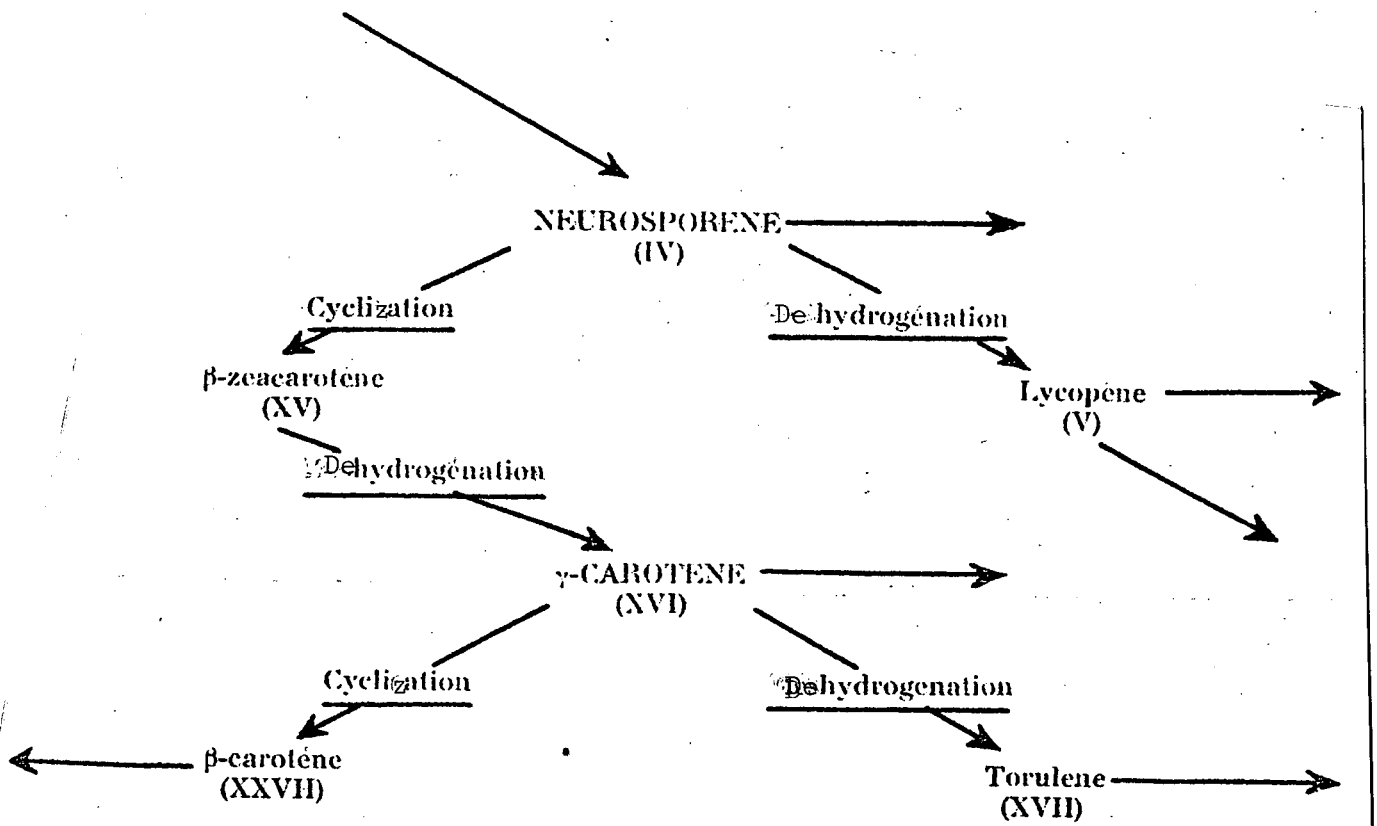
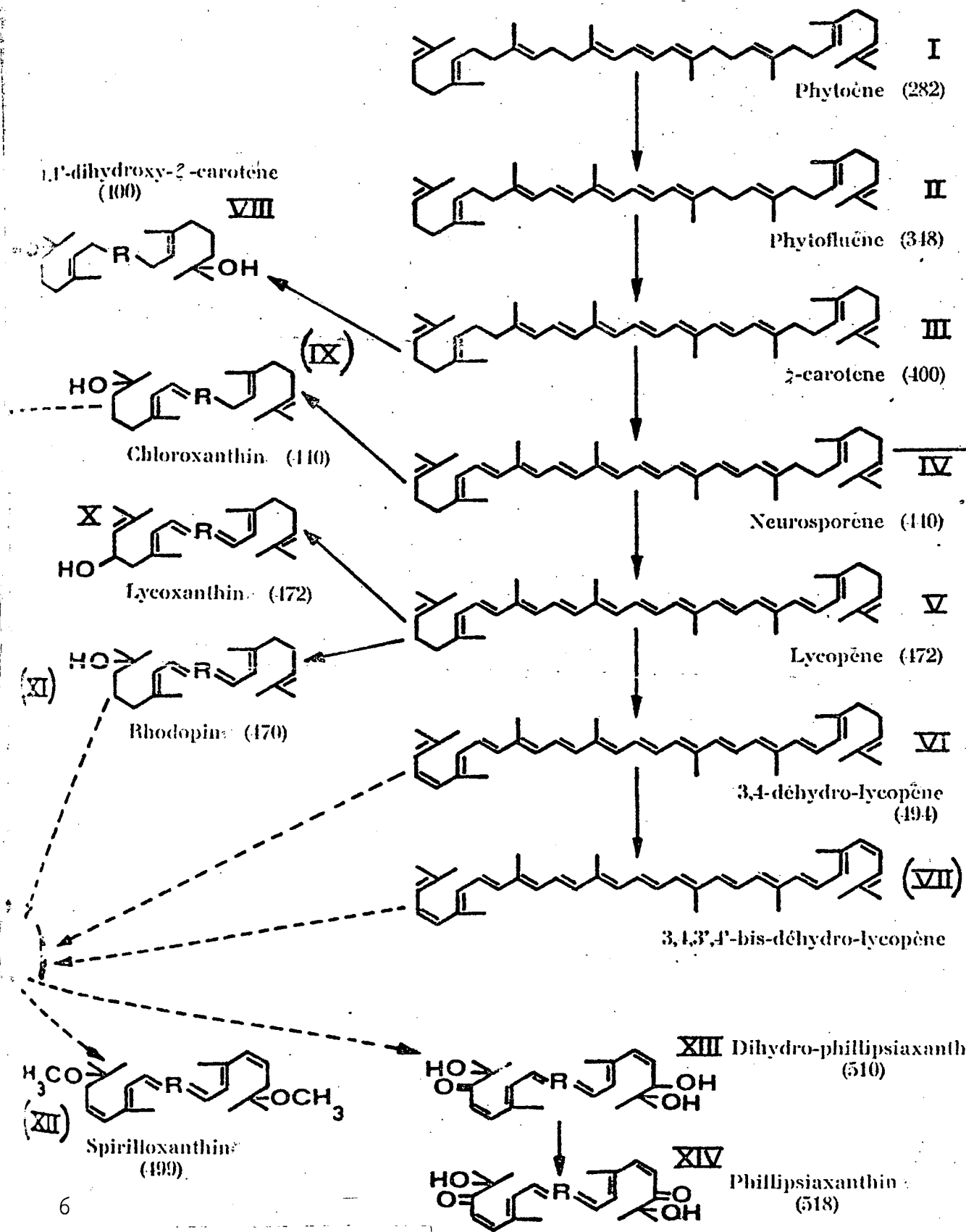

NEUROSPORENE
(IV)
Cyclization
Dehydrogénation
β-zeacaroténe
(XV)
Lycopène
(V)
Dehydrogénation
γ-CAROTENE
(XVI)
Cyclization
Dehydrogenation
β-caroténe
(XXVII)
Torulene
(XVII)

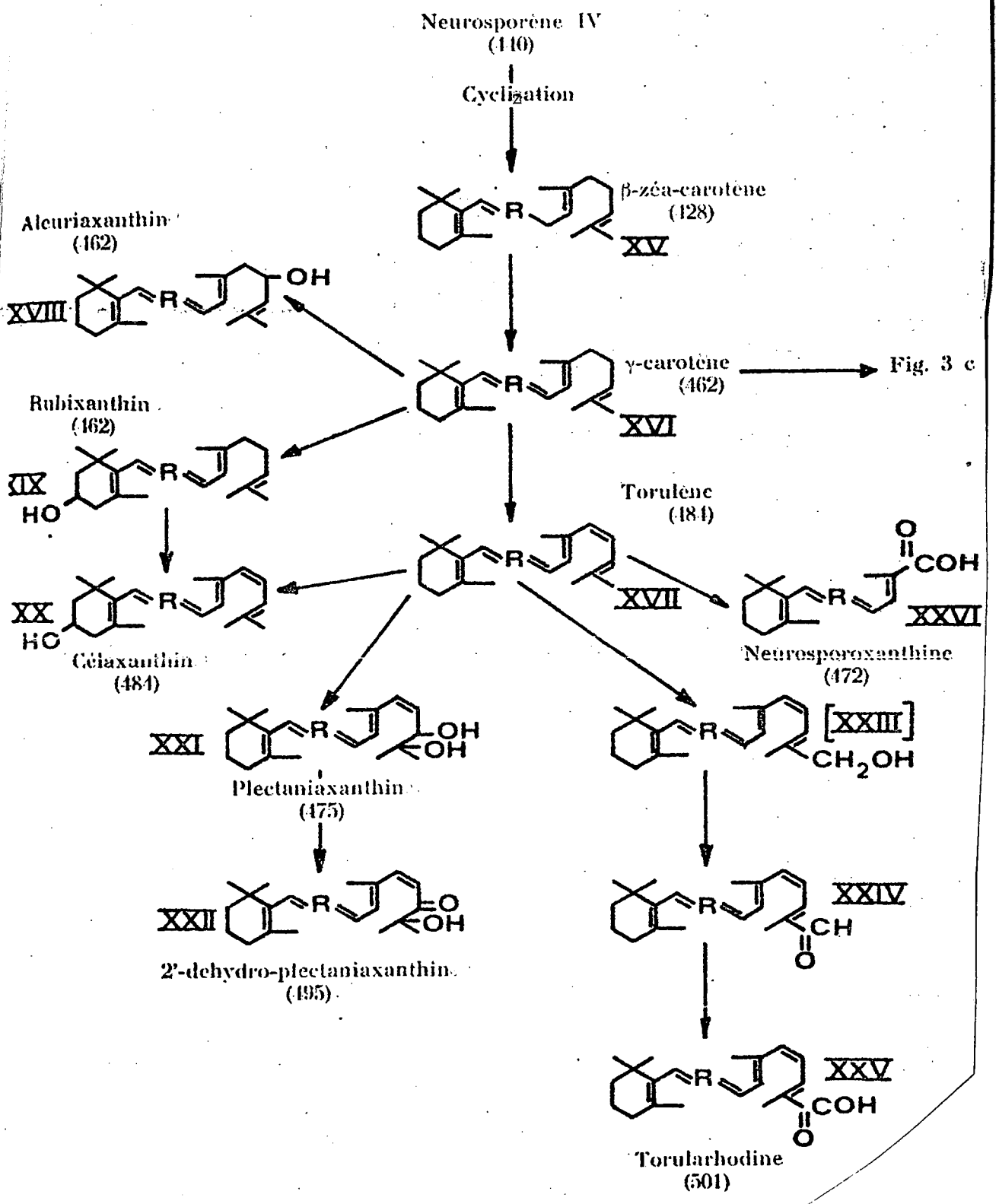
Fig. 2



ALIPHATIC CAROTENOIDS

Fig. 3 a

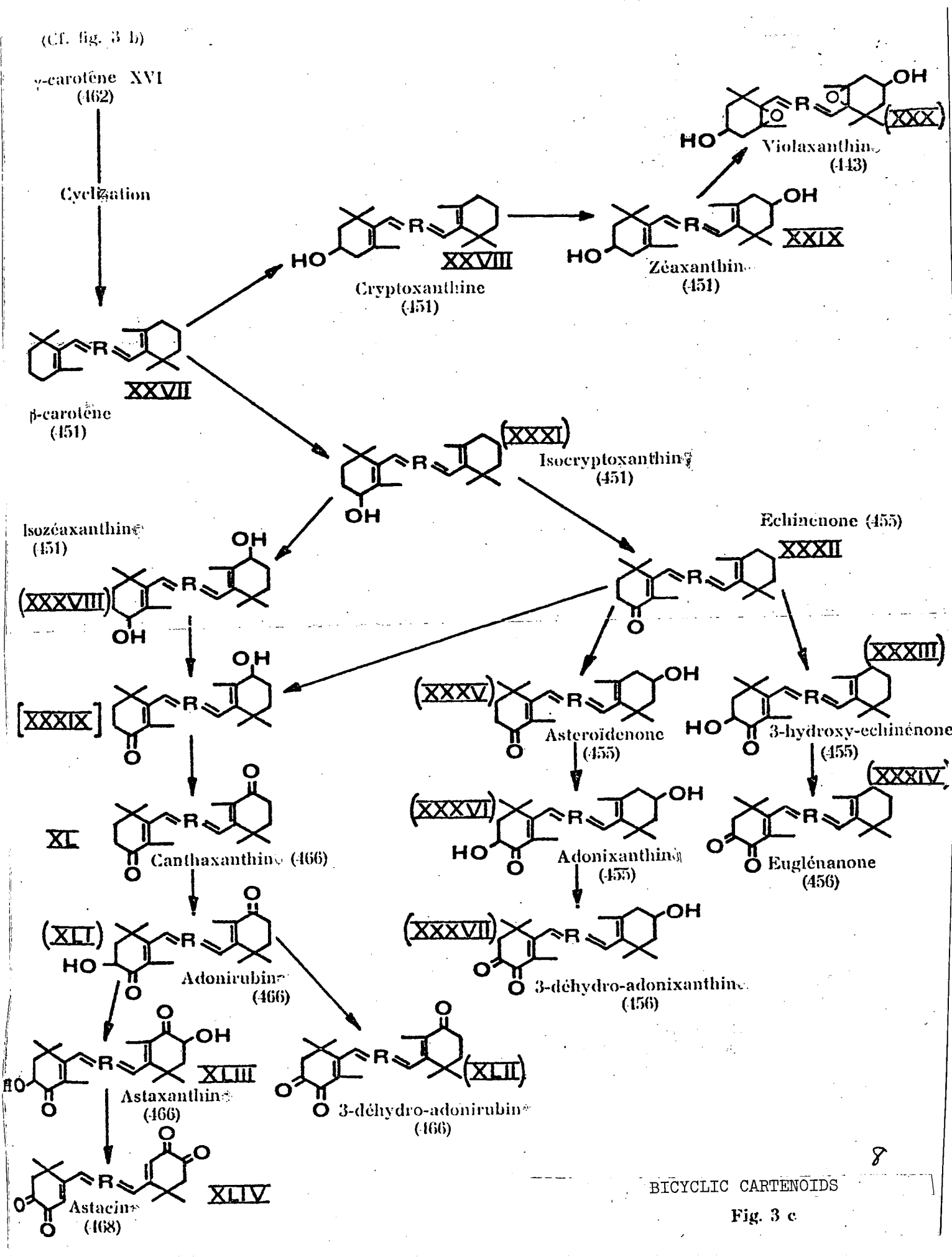
(Cf. fig. 3 a)



MONOCYCLIC CAROTENOIDS

Fig. 3 b

(Cf. fig. 3 b)



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BICYCLIC CARTENOIDS

Fig. 3 c

and oxygenation of the molecule. Oxygen can appear in the form of a hydroxyl group (primary alcohol: lycoxanthin*) secondary: cryptoxanthin XXVIII; tertiary: chloroxanthin IX), carbonyl /5
(aldehyde: torularhodin XXIV aldehyde; ketone: echinenone XXXII) or carboxyl (torularhodin XXV); methoxyl groups of the carotenoids of many crimson photosynthetic bacteria (spirilloxanthin XII) and epoxides which are so frequent in the xanthophylls of green plants (violaxanthin XXX) seem here to be unknown. We will add that fungus xanthophylls can exist in a free or esterified form.

2. DISTRIBUTION OF CAROTENOIDS IN FUNGI

The work done in this field, the results of which are recapitulated in the tables below, are still somewhat scanty in relation to the broad scope of the problem, and much remains to be done before general and final conclusions can be drawn. Moreover, such research is often handicapped by the scarcity of the available starting material; the pigment content is often low, and quantitative extraction has sometimes proved difficult; thus we are led to believe that in most of the analyses to date, especially the older ones, only the major compounds were detected. However, present-day paper and thin-layer chromatography techniques, together with characteristic reactions on the microchemical scale and the increasing number of reference compounds synthesized by organic chemists, permit identification or even structural analysis of isolated pigments in tiny quantities [43, 9, 10]. The above permits us to hope that long strides will be made in our knowledge of this field.

The presence of carotenoids in many fungus species is known from the work of Bachmann [12], Zopf [167], Kohl [95], and Van Wisselingh [163]; in many cases a structural study remains to be done. Just as valuable to the taxonomist are the lists prepared by Zopf [167] and other authors after him [23, 119, 71] of species not producing carotenoids although their color has often suggested these pigments. It should be noted that, conversely, the presence /6
of carotenoids can be masked by the dominant presence of compounds showing another color, for example green in Leotia lubrica [167]. The absence of vitamin A activity [66] does not permit us to conclude that carotenoids are absent since many of them (not containing the free "β" cycle) are inactive from this point of view. On the other hand, their location in the cell has enabled us to suspect the presence of carotenoids in many species [78-82, 84].

Research with the aid of modern methods of analysis into fungus carotenoids has been carried on here and there for three decades either with the purpose of deepening knowledge of one

particular species, usually with brilliant coloring, or with a physiological intent, but also, sometimes, for taxonomical reasons: we will cite the work of Cantino and Hyatt [25], Turian [147], and Valadon [150, 152, 154].

The results of these various studies are assembled in Tables I through VI; but the reader should have no illusions about them. First, of some 100,000 species of fungus described, only about a hundred have been studied from this viewpoint; even though the majority of fungi are not carotenogenic, we can see how much of the present data are still partial. Second, many analyses are already quite old and should be re-performed with modern methods; even recent identifications have proved erroneous [107]; several species have been analysed serially (Allomyces javanicus, Cantharellus tubaeformis, Sphaerobolus stellatus, etc.) and each author has corrected or completed the contributions of his predecessor due to the thorough-going transformation of techniques over the last decade. Finally, as we have already said, in many cases the presence of carotenoids has been simply noted, without their identification having yet been made.

We have not mentioned here the α-carotene, reported in some rare species of fungi in now-ancient studies. Very close to the β-carotene (a double bond at 4-5 instead of 5-6), relatively difficult to distinguish from certain cis isomers of this unless very pure, this pigment of green plants has never been found in recent analysis of species from which it had formerly been thought to have been isolated. The same applies to δ-carotene, thought to be present in Neurospora crassa [74] and the hypothetical θ-carotene of N. crassa and N. sitophila [76]. Although recent results [121] argue in favor of the natural existence of lycopersene (different from phytoene I, of which it could be the precursor, by the absence of a central double bond) the argument for its presence in N. crassa [68] has subsequently been weakened [37]. /7

3. CHEMICAL, TAXONOMIC, AND PHYLOGENETIC CONSEQUENCES

3.1 Singularities of Fungus Carotenogenesis

The nature and distribution of carotenoids in fungi distinguish them most clearly from green plants; although there are resemblances with certain algae and animals (those not building the hydrocarbon skeleton of the carotenoids but which, in certain groups, can cause it to undergo progressive oxidation), the carotenogenesis of fungi has its own particularities.

TABLE I

	Presence of Carotenoids	Neurosporene (IV)	Lycopene (V)	Dehydro-3,4 lycopene (VI)	γ-carotene (XVI)	Torulene (XVII)	Neurosporo-xanthin (XXVI)	β-carotene (XXVII)	Zeaxanthin (XXIX)	References
MYXOMYCOTINA										
MYXOMYCETES										
Lycogala epidendron			+	+	+	+	+	(+)		101,107
L. flavofuscum	+									167,95
Stemnitis ferruginea	+									167
EUMYCOTINA										
PHYCOMYCETES										
Chytridiales :										
Chytridium spp.	+									167,95
Rhizophlictis rosea			+		(+)		?			35
Cladochytrium replicatum			+		(+)					56
Pleotrachelus fulgens	+									167,95
Blastocladiales :										
Blastocladiella spp.					(+)					25
Allomyces arbuscula					(+)					45
A. macrogynus					(+)					45,148
A. moniliformis					(+)					45
A. javanicus			+					+		a45,149
A. cystogena	-									45
ZYGOMYCETES										
Blakeslea trispora					+			(+)	?	b4,30,153
Choenophora cucurbitarum								(+)		13
Mucor flavus	+									95,163
M. hiemalis								(+)		69
Phycomyces blakesleeanus		+	+		+			(+)		c58,59,38,161
P. nitens		+	+		+			(+)		d65
Pilobolus kleinii								(+)		24
Pi. crystallinus	+									167,95
Pi. oedipus	+									167,95

Also identified:

a. : Phytoene (I), Phytofluene (II), ζ-carotene (III).
b. : Phytoene, Phytofluene, ζ-carotene, β-zeacarotene (XV).
c. : Phytoene ?, Phytofluene, ζ-carotene, β-zeacarotene.
d. : Phytoene ?, Phytofluene, ζ-carotene.

Key:

- : absence
+ : presence
? : result doubtful
(+) : major constituant

TABLE II

	Presence of carotenoids	Neurosporene (IV)	Lycopene (V)	Lycoxanthin (X)	Dehydro-3,4 lycopene (VI)	Dihydro-2'phillipsiaxanthin (XIII)	Phillipsiaxanthin (XIV)	γ-carotene (XVI)	Aleuriaxanthin (XVIII ?)	Torulene (XVII)	Celaxanthin (XX)	Plectaniaxanthin (XXI)	Dehydro-2' plectaniaxanthin (XXII)	Torularhodin (XXV)	Neurosporoxanthin (XXVI)	β-carotene (XXVII)	Xanthophylls not identified	Acid carotenoids not identified	References
EUMYCOTINA (cont.)																			
ASCOMYCETES																			
Endomycetales :																			
Sacchararomyces sp.	+																		122
Zygosaccharomyces spp.	-																		119
Pichia spp.	-																		119
Aspergillales :																			
Aspergilius niger																		(+)	165
A. giganteus																(+)			57,168
Penicillium sclerotiorum																(+)			118,116
P. multicolor																+			a117
P. lapidosum																	(+)		117
Rhizoctonia zeae																(+)			b117
Protomycetales :																			
Protomyces innundatus			?					+								(+)			150
P. incuyei			+					+								(+)			152
P. pachydermus			+					+								(+)			152
Taphrinales :																			
Taphrina deformans	-																		152
T. communis	-																		152
Clavicipitales :																			
Claviceps purpurea	-																		167
[one word illegible] typhina			?													(+)			147
[one word illegible] militaris																	(+)		55

Also identified:

[two lines illegible]

TABLE III

	Presence of carotenoids	Neurosporene (IV)	Lycopene (V)	Lycoxanthin (X)	Dehydro-3,4 lycopene (VI)	Dihydro-2,phillipsiaxanthin (XIII)	Phillipsiaxanthin (XIV)	γ-carotene (XVI)	Aleuriaxanthin (VIII ?)	Torulene (XVII)	Celaxanthin (XX)	Plectaniaxanthin (XXI)	Dehydro-2',Plectaniaxanthin (XXII)	Torularhodin (XXV)	Neurosporexanthin (XXVI)	β-carotene (XXVI)	Xanthophylls not identified	Acid carotenoids not identified	References
EUMYCOTINA (cont.)																			
ASCOMYCETES (cont.)																			
Pezizales :																			
Pyronema confluens								+		+	+			+		+			26
Ascobulus spp.	+																		167
Humaria ssp.	+																		78, 80
Scutellinia scutellata								(+)		+							+		107
S spp.								(+)		+							+		6
Melastiza miniata	+																		80
Anthracobia melanoma	+																		80
Saccobolus violaceus	-																		167
Peziza bicolor	+																		167, 95
P. aeruginosa	+																		167
Aleuria aurantia			+					(+)	+				+			+	+		107, 6
Plectania coccinea												(+)	+			+			9
Phillipsia carminea			+			+	(+)						+						10
Cookeina sulpices							(+)							+					11
Helotiales :																			
Helotium citrinum																(+)			147
Leotia lubrica	+																		167
Spathularia flavida	+																		167, 95
Dasyscypha bicolor	+																		123
Erysiphales :																			
Oidium violaceum	-																		167
Sphaeriales :																			
Sphaerostible coccaphili	+																		163
Neurospora crassa		+			+			+		+					+	+			a74, 107, 108
N. sitophila		+	+		+			+		+					+	+			a74, 3, 107
Polystigma fulvum	+																		167
P rubrum				?															101
Nectria cinnabarina	+																		167, 163

Also identified:

a. : Phytofluene (II), ζ-carotene (III).

TABLE IV

	Presence of Carotenoids	Lycopene (V)	γ-carotene (XVI)	Rubixanthin (XIX)	Torulene (XVII)	β-carotene (XXVII)	Cryptoxanthin (XXVIII)	Xanthophylls not identified	Acid carotenoids not identified	References
EUMYCOTINA (cont.)										
BASIDIOMYCETES										
Heterobasidiae										
Calocerales :										
Calocera viscosa		+				(+)	+			147
C. cornea	+									163
C. palmata	+									163
Dacromyces stellatus			+		+	(+)	+			a 61
D. ellisii		?	?			(+)				70
Tremellales:										
Ditiola radicata	+									167, 95
Tremella mesenterica						(+)				101
Guepinia helvelloides	-									147
Uredinales :										
Coleosporium senecionis			+	+		+				101
C pulsatilla	+									15
C. euphrasiae	+									167, 95
Gymnosporangium juniperinum	+									12
G. juniperi-virginianae			(+)			+		+		138
Kunkelia nitens			+			+				29
Melampsora aecidiodes	+									12, 15
M. salicicapreae	+									12, 15
Phragmidium violaceum	+									167
Puccinia coronata	+									12
P. coronifera			+			+			+	101
P. graminis		+	+		+	+	+			b 86
Triphragmium ulmariae	+									12, 15
Uredo aecidiores	+									167
Uromyces alchemilliae	+									167, 95

These have also been identified:

a. : Phytoene (I), Phytofluene (II), ζ-carotene (III).

b. : Phytoene.

TABLE V

	Presence of Carotenoids	Neurosporene (IV)	Lycopene (V)	β-carotene (XVI)	β-carotene (XXVII)	Echinenone (XXXII)	Canthaxanthin (XL)	Astaxanthin (XLIII)	Astacin (XLIV)	Keto-carotenoids not identified	References
EUMYCOTINA (cont.)											
BASIDIOMYCETES (cont.)											
Homobasidiae											
GASTEROMYCETES											
Nidulariales :											
Sphaerobolus stellatus				(+)	+						54, 132
Phallales :											
Anthurus aseroiformis		+	(+)								41, 146
Clathrus cancellatus	+										78
Lysurus hexagonus	+										78
Mutinus bambusinus	+										78
M. caninus				+	+						46
Pseudocolus spp.	+										78
HYMENOMYCETES											
Aphyllophorales :											
Peniophora aurantiaca					+					(+)	8
Polyporus spp.	-										23
Polysticus spp.	-										23
Trametes spp.	-										23
Daedalea flavida	-										23
Lenzites subferruginosa	-										23
Ganoderma lucidus	-										23
Clavaria cardinalis	+										84
Cantharellus cinereus	-										147
C. tubaeformis		(+)	+								a162,147,51
C. lutescens		+	(+)								b162,147,51
C. cibarius			+	+	(+)						162
C. cinnabarinus					+		(+)			+	c75
Agaricales :											
Clitocybe venustissima				(+)	+						5
Hygrophyropsis aurantiaca	-										5
Agaricus laccatus	-										167
Cortinarius violaceus	-										167
Lactarius torminosus (mycelium)					+						91

These have also been identified: a. : Phytofluene (II), ζ-carotene (III), Dihydroxy-ζ-carotene (VIII).
b. : Dihydroxy-ζ-carotene ? c. : Phytofluene ?

TABLE VI

EUMYCOTINA (cont.) DEUTEROMYCETES	Presence of Carotenoids	Neurosporene (IV)	Lycopene (V)	Dehydro 3,4 lycopene (VI)	γ-carotene (XVI)	Torulene (XVII)	Torularhodin (XXV)	Neurosporo-xanthin (XXVI)	β-carotene (XXVII)	Xanthophylls not identified	References
Arthrobotrys oligospora					+	+		?	(+)		154
Epicoccum nigrum					+		+		+	(+)	67
Fusarium aquaeductum		(+)			+			?			130
F orobonchus	+										16
Verticillum albo atrum		+	+		+	+		?	+		a 155,156,157
Sporidiobolus johnsonii					+	+	(+)		+		49
Sporobolomyces roseus						+	(+)		+		101, 17
S salmonicolor						+	(+)		+		101
S ruberrimus					?	+	(+)		+		164
Rhodotorula glutinis		+			+	+	(+)		+		a120,126,134,136
Rh rubra		+			+	+	(+)		+		b 53, 22,120, 14
Rh sanniei			?		+	+	(+)		+		143
Rh mucilaginosa		+	+	?	+	+	(+)		+		c 158, 21
Rh aurantiaca					+				+	(+)	120, 21
Rh minuta					+	(+)	+		+		120,126
Rh gracilis					+	(+)			+		d 128
Rh infirmoniniata					+	(+)			+		120
Rh pallida					+	(+)			+		120
Rh aurea			+		+				(+)		120
Rh flava			+		+				(+)		120
Rh peneaus			+		+				(+)		120
Cryptococcus flavescens			+		+				(+)		120
C. laurentii			+		+				(+)		120
C. luteolus			+		+				(+)		120
C. albidus	+										71
C. diffluens	+										71
C. neoformans	-										71
Candida spp.	-										119
Torulopsis spp.	-										119, 14, 71
Trichosporon spp.	-										71
Trigonopsis spp.	-										71
Pitysporum spp.	-										71
Brettanomyces spp.	-										71
Kloeckera spp.	-										71
Pullularia spp.	-										119

These have also been identified:
a. : (I), (II), (III), (XV).
b. : (II), (III).
c. : (I), (II), (III), (XV), (XXIV).
d. : (I), (II).

The first fact which needs to be brought out is the absence of carotenoids in many groups, while these pigments are universally present in the rest of the plant kingdom and exist in the biosphere even before the cell structure of the eucaryotes: indeed the pigments of photosynthesis always include carotenoids-xanthophylls in particular side by side with the chlorophylls. The loss of carotenoids in certain groups of fungi thus appears as a secondary characteristic, authorized by the loss of autotrophy.

While the carotenoid content of the chlorophyll tissues of green plants shows remarkable uniformity where β-carotene and lutein (dihydroxy-3, 3 α-carotene) predominate inside an ever-complex mixture, the fungi can accumulate only one pigment: this can be β-carotene (XXVII) but also γ-carotene (XVI) or lycopene (V). This characteristic relates them closely to the non-chlorophyllaceous parts of green plants, particularly flowers and fruits. /8

Similarly, hydroxycarotenoids of fungi are very frequently accumulated in the form of esters which, in green plants, is the case for non-chlorophyllaceous tissues [63] and, in a single alga cell (Acetabularia mediterranea) we find together xanthophylls "of photosynthesis" in a free form and esterified hydroxyketo-carotenoids [44].

But it is mainly by the nature of the pigments formed that fungi are characterized. The extreme rarity of xanthophylls in Phanerogames - lutein in particular has never been found to our knowledge - has been noticed for a long time in fungi; indeed oxygenated carotenoids are not uncommon, but their metabolism takes place along different paths, sometimes original. Although fungi do not seem to have used the α-carotene path, they are the only eucaryotes to have pushed in the direction of monocyclic carotenes beybnd γ-carotene; they have no epoxycarotenoids, but acid carotenoids with C_{40} are their characteristic feature; hydroxylation at 3 is rare in this group, but they are also the only eucaryotes to hydrolyze carotenoids at 1 and substitute them at 2; keto-4 carotenoids have been shown only recently in higher plants [33] and, as their names indicate, they were at first mainly isolated from animals (echinenone XXXII, asteroid-enone XXXV, astaxanthin XLIII), then fungi (canthaxanthin XL) and algae (euglenanone XXXIV). The synthesis capabilities proper to fungi have not been characteristic of the entire taxon, but generally of a limited group and a high level of evolution; no type of skeleton, no method of substution is universally represented; the only common characteristics that we can find are,

here too, negative: absence of epoxylated derivatives, great uncertainty as to the presence of the α cycle, rarity of hydroxylation at 3 occurring alone. However, each of these characteristics separates the fungi from most of the plant phyla, and they are not found together in any chlorophyll group.

We can now see to what extent the carotenogenis of fungi is distant from that of chlorophytes and chlorophyllaceous plants in general. No longer linked to photosynthesis it was able either to disappear or to go off in directions which, for the same reason, are found in the flower and fruit, or forge further ahead in oxidation, rejoining animal metabolism, but also and above all plunge into quite original paths here and there. The xanthophylls disappeared with photosynthesis, with which they are more or less linked, which would explain the singularities of hydroxylation of fungi, which reappeared secondarily. Similarly, the aliphatic and monocyclic pigments, in the general case mere intermediaries in the synthesis of bicylics, were able to accumulate here, desaturation of their skeletons then being advanced further (dehydro-3, 4 lycopene, torulene and derivatives) than in the green plants. /9

3.2 Taxonomic Implications

While carotenoids in the algae permitted taxonomists to distinguish the great phyla and define their affinities [64] they are not as widespread in the fungi nor sufficiently well known to be able to play such a role at this time. At most, within the septomycetes, we can say that the path of the monocylcic carotenoids seems more characteristic of ascomycetes; here indeed is the most original path taken by fungus carotenoids, and the richest. The basidiomycetes appear rather to accumulate aliphatic or bicyclic carotenoids; pigments belonging to other biogenetic families tend to predominate here, in particular in the higher groups.

Considering carotenoids has proved useful in the middle taxonomic units. Thus, for example, within the Tremellaceae, the separation between Tremellales and Guepiniales has been confirmed by the nature of pigmentation, the former appear carotenogenic and the latter quinonogenic [147]; a difference of the same order seems to separate, in the Sarcoscyphaceae, Sarcoscyphae and Urnulae [6]. No doubt, carotenoids could render further such services to the taxonomist in the Heterobasidiae and the Aphyllophorales, for example.

Carotenoids have proved valuable for classifying Deuteromycetes, with poor morphology lacking the essential criterion of sexual forms of reproduction. Thus, Cryptococcaceae and Rhodo- /10

torulaceae are distinguished by the manifest carotenoid pigmentation of the latter [112]. Most Cryptococcus have in their turn been shown to be carotenogenic [120, 126, 71], and Hasegawa et al have classified those with this character with the Rhodotorula thus divided, according to the kind of their carotenoids, into Flavotorula and Rubrotorula; but on the basis of other criteria, Rh. aurantiaca has been classified in the second sub-genus despite the singularities of its pigmentation [72, 73]. Similarly, Rhodotorula had for a long time been classed with Sporobolomyces since they have a group of characters in common, including coloration; analysis has validated this viewpoint by showing that the same complex of carotenoids is involved in both cases [101, 17, 164] and these conclusions have been brilliantly confirmed by immunological studies [145]. Similarly the identity of their pigmentation supports the close affinity of the genera Sporobolomyces and Sporidiobolus [49]. But knowledge of their carotenoids could also permit, in some cases, specification of the relationships between imperfect fungi with the groups whose sexuality is known. Work in this direction has been undertaken for nematophagous Deuteromycetes [154] and better knowledge of the pigments of Heterobasidiae will perhaps permit the relationship, considered by many authors, that carotenogenic yeasts might have with them to be better defined.

Also, within the Eumycetes, carotenoids can permit one genus to be restricted and the position of a species to be defined. A system unit is sometimes characterized by constant pigmentation: thus Scutellinia scutellata and Sc. arenosa have an exactly identical carotenoid content [6]. The general absorption spectrum of solution in ether-petroleum is then characteristic; elsewhere two sub-genera have been defined, as in the above-described case of Rhodotorula. The presence alone of carotenoids can be a decisive factor, as in the above-cited case of Clitocybe venustissima; we should however be careful to state that total or partial absence of carotenoids must be considered with prudence by the taxonomist: the classic example of spontaneous or experimental mutations (often regressive) are an initial index for this. Indeed, alteration of just one gene can entail impoverishment or /11
loss of carotenogenesis; but on the other hand new carotenoids have sometimes appeared for the species, even a rich and varied carotenogenesis in a species normally colored by quite different compounds [155]. It is difficult to imagine that the genes necessary for a whole biosynthetic chain would appear de novo; it is more likely that they already existed in a latent form and have simply been revealed by mutation. In this connection it is significant that in the case where carotenoid synthesis has

suddenly been manifested, phytoene, a colorless precursor of carotenoids sensu strictu (and which accumulates when their biogenesis is inhibited) was already present [155]. Also, we should not forget, especially with species cultivated in the laboratory, the preponderant influence of environmental conditions on pigment formation in fungi. The actual potentialities of a group, whether manifest or hidden, all interest the taxonomist; thus in the present case he must exercise caution before attributing an absolute value to some criterion, in particular a negative one, and must compare the contribution with the more classical data.

3.3 Application to Phylogenesis

After helping to determine the system groups and defining their affinities, Comparative Biochemistry will be able to play an outstanding part in establishing their evolutional relationships. Indeed, if we find that compounds A, B and C arise biochemically according to the sequence A-B-C, we may postulate their appearance in the same order during biological evolution. Analysis thus furnishes an observation on evolution which is not only directional but literally quantified. Thus, the Sarcoscyphaceae, rich in "terminal" pigments (plectaniaxanthin XXI, phillipsiaxanthin XIV) can be considered as the furtherest evolved of the Discomycetes, and this accords with cytological and biological data. We shall note in passing that the color alone is an initial index, although rough, of the relative levels of evolution of pigments belonging to a given chemical family, and thus the related organisms which accumulate them. Indeed, evolution along a biogenetic chain of pigments occurrs most commonly by progressive oxidation*; now this (desaturation, appearance of C = 0 groups) often leads to lengthening the chromophore system of double conjugated bonds, which is translated by displacement of the absorption spectrum, called bathochrome, by a shift of the color towards the red end of the spectrum: the first carotenoids (phytoene I, phytofluene II) absorb only in the near UV (from 282 to 348 nm respectively in an ether-petroleum solution) and thus have no color; neurosporene (IV, λM 440 nm) gives lemon yellow solutions, lycopene (V, λM 472 nm) is the pigment of the tomato and phillipsiaxanthin XIV, λM 518 nm) is clearly violet. This rule is not, evidently, of universal application: on the contrary /12

*The same direction of evolution had already been postulated by Lebreton [100] in the case of flavonoids and it seems that it could be extended further. Note that evolution also proceeds in the direction of increasing entropy, obeying the well-known law of thermodynamics.

cyclization and hydration of a double conjugated bond have a hypsochrome effect; but, within certain limits, this simple character is not without value. Moreover, for one and the same qualitative composition, the proportions of the various pigments can permit a divergence in evolutional trend to be distinguished: of two fungi containing β- and γ-carotenes, that in which β-carotene accounts for most of the pigmentation is clearly directed along the path of the bicyclic skeleton, which, on the contrary, seems to be abandoned to the advantage of that of the monocyclic carotenes in the species in which γ-carotene predominates. Thus, two Geoglassaceae - Leotia lubrica (β-carotene 81% of total pigmentation, γ-carotene 12%) and Microglossum olivaceum (β-carotene 23% and γ-carotene 73%) [6]. The results obtained by N. Arpin permit the Discomycetes to be considered as a group to which this kind of reasoning applies particularly forcibly.

However, application of this principle of recapitulation of phylogenesis by biogenesis - here a fully justified renaissance /13
of Haeckel's Law, where superposition of chemical and biological evolution permit the latter to be analyzed by the former - must be carried out cautiously: a phyletic derivation can just as well be accompanied by the loss as by the gain of biogenetic capacities and, going through chains of reaction, evolution can here "go backwards"* Moreover, do not fungi as a whole derive from chlorophyll organisms, and are they thus not all carotenogenic? According to their distribution, we can approximately fix for lycopene, γ-carotene and β-carotene the limits of the original "holdings" of the fungi and, just as much as its development, the disappearance of this family of pigments is here a fact of evolution. Here is an example where biosynthetic and phyletic chains seem actually opposed: in the Rhodotorula, probably from γ-carotene, the Rubrotorula accumulate torularhodin (XXV), Flavotorula mainly β-carotene (XXVII); pigmentation of the latter thus appears more "primitive." However, torularhodin exist in species related but morphologically less specialized (Sporidiobolus, Sporobolomyces); it thus appears, and many other characters show this, that "yellow" carotenogenic yeasts derive from the "red" yeasts by loss or inhibition of a number of capacities [72, 73, 145].

Under the auspices both of the Mycology Laboratory associated with the National Center for Scientific Research and the Faculty of Sciences, Lyons Phytochemistry and Phytophysiology

*Here we can find the classical opposition between often regressive microevolution of a structure (here chemical, not morphological or anatomical) and macroevolution creating new structures.

Section of the Department of Plant Biology, a team was formed to study (taxonomically for the moment) products of fungal metabolism, especially pigments. Since N. Arpin is presently concentrating on Discomycetes, a vast group rich in varied and often original carotenoids, we decided to undertake this "overview" of carotenogenesis in Basidiomycetes. Apart from the interest inherent in its general nature, such a study would mark off the system groups where detailed research into carotenoids would be particularly promising from the taxonomic point of view, and would perhaps detect a species particularly propicious to later physiological studies of this family of pigments. /14

CHAPTER II

MATERIALS AND TECHNIQUES /15

1. BIOLOGICAL MATERIAL

1.1 Cultures

Organisms cultured in the laboratory, unless otherwise specified, were prepared after various tests according to the following procedure: a preculture of the strain studied is made in Wickerham's liquid glucosed medium [160] (tubes used for assimilation tests upon yeast determination [20] placed in the oven under agitation at a temperature of 25°C). After three days' development, 2 cm^3 of this suspension seed 500 cm^3 of liquid culture medium contained in a vertical cylindrical container with capacity 1.25 liters, permitting sterile air to be bubbled through. This culture medium is made up as follows:

Massed glucose	20 g
"Moser" maltea	2 g
$MgSO_4$, 7 H_2O	1 g
KH_2PO_4	0.5 g
$(NH_4)_2SO_4$	0.5 g
H_2O quant. suff. for	1 liter

As already noted [72], purely synthetic culture media often produce, at least in the case of carotenogenic yeasts, a fairly mediocre pigment. The culture medium chosen here furnishes as good results as the yeast and potato culture [72, 73] while being incomparably easier to prepare.

The temperature is held at 25°C, the cultures receive 200 lux white light for 6 h. daily. After 9 days' growth, yeasts or hyphae are assembled and washed by centrifugation.

1.2 Provenance /16

The carpophores collected wild come, unless indicated to the contrary, from the nearby surroundings of Lyons.* As quickly as possible, these samples are carefully cleaned and placed in acetone. In the case of epixyls—especially the encrusted ones—the fructifications were carefully removed; some slight chlorophyll contamination (algae, etc.) from the fungal material is, however, inevitable in this case, despite every care taken to remove it.

1.3 Preservation

In all cases the material thus obtained is preserved in acetone in the freezer until analyzed; by dehydrating it this solvent prepares for later extraction of the lipophile pigments.

2. DETERMINATION OF PIGMENTS

2.1 Relationships between Chemical Structure and Physical Properties

The present general method for separating and characterizing carotenoids uses essentially distribution of the pigments in two phases (partition of non-miscible solvents and adsorption chromatography) and their spectral properties, first that of absorption in the visible range. We will first briefly recall the relationships established between the chemical structure of the various carotenoids and their properties in these domains.

2.1.1. Relationships between partition and structure. Analysis of a complex mixture of carotenoids for a long time began by dividing it between two non-miscible solvents: usually hydrocarbon and acqueous alcohol. According to the principle of *similia similibus solventur* the hydrocarbons solubilize in the epiphase while the xanthophylls are easily soluble in alcohol. In a more complicated method [36] the petroleum-ether solution can first be extracted several times by 90% methanol, which takes up the polyhydroxylated xanthopylls; the other carotenoids remain in the epiphase which is then placed with 95% methanol which takes up the monohydroxylated carotenoids. Such a process is presently being used less and less /17 since the development of chromatographic techniques and the intermediary solubility of many natural carotenoids, which are thereby

*We would like to thank Mr. Bussy, Vice Chairman of the Societe Linneenne de Lyon, who procured samples of often rare species for us.

found in the different phases. However, it is still interesting in the case where one of the pigments present possesses very special solubility (acid and glycoside).

Measurement of the partition coefficient of an isolated pigment is a useful criterion of characterization and structural determination. Petracek and Zechmeister [127] measured, under specified conditions, partition of a number of carotenoids between hexane and methanol (95% or 85%). The behavior in the first system of the main representative types is shown in Table VII. Hexane can be replaced by petroleum ether (boiling point 40-60° C) usually employed in carotenoid chemistry, with no overall change in the results [103].

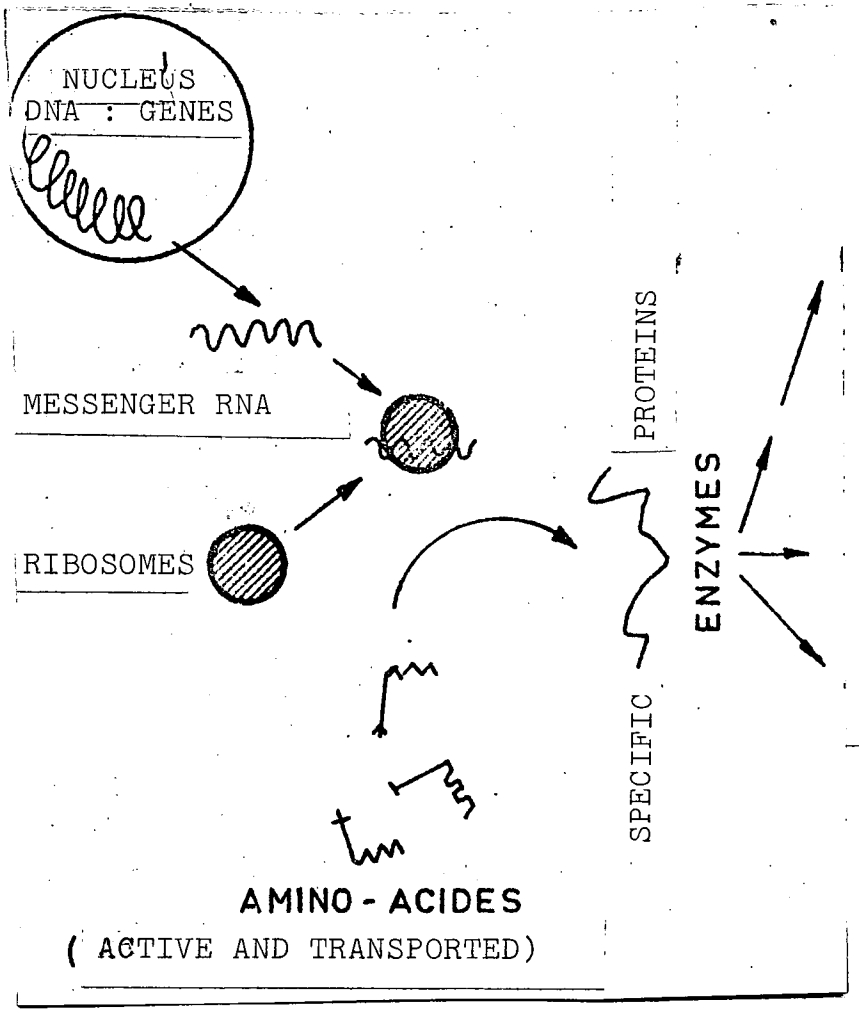
Krinsky [196] chose as an index of carotenoid hydrophily the methanol concentration as the pigment divides equally between the two phases, or "M_{50}"; he observes that between certain limits there is a linear relationship between the coefficient partition and the degree of hydration of the methanol used. According to this author, hydrophily of a carotenoid is very substantially the sum of the actions proper to each of the radicals it carries; and taking as unity hydrophily of a non-allylhydroxyl, he attributes the following relative values to the various functions of the pigments studied:

Epoxide 5.8	0.24
Epoxide 5.6	0.24
Acetic ester	0.47
Ketone	0.72
Allylhydroxyl	0.89

An abacus will convert, to this unit, the M_{50} of a carotenoid determined directly or by interpolation on a graph from several measurements in different systems. It should however to be noted that, if neither the isolated double bonds nor the nature of the hydroxyls significantly influence the partition coefficient, the hypophasic characteristic of an aliphatic xanthophyll increases with the length/18 of its conjugated polyenic chain [103].

The results obtained by Krinsky have, as a whole, been confirmed by Subbarayan et al [140].

Determination of their partition coefficient is thus an interesting method of structural analysis—number, nature, and position of radicals—of the carotenoids, provided their hydrophily is not too high [111]. What is more perhaps, modification of this characteristic after this or that characteristic reaction (acetylation,



CRITERIA DISCIPLINE: SYSTEMA-TICS

SYSTEMATIC:

MORPHOLOGICAL----------

ANATOMICAL----------

CYTOLOGICAL----------

CARYOLOGICAL

BIOLOGICAL

PHYSIOLOGICAL----------

IMMUNOLOGICAL-SEROLOGY----

BIOCHEMICAL-CHEMOTAXONOMY

BIOTAXINOMY

CLASSICAL

MODERN

TOTAL

Table VII

Partition coefficients of various pigments between petroleum ether and methol 95% (according to Petracek and Zechmeister [127]).

Hydrocarbons		100 : 0
Dipalmitate of zeaxanthine	2 —O— $\overset{\overset{O}{\|}}{C}$ — $C_{17}H_{35}$	100 : 0
Ethoxy-4 *-carotène	1 -O-C_2H_5	99 : 1
Echinénone (XXXII)	1 =O	93 : 7
Diacétate of isozéaxanthine	2 - O - $\overset{\overset{O}{\|}}{C}$ — CH_3	86 : 14
Isocryptoxanthin (XXXI)	1 —OH (allyl)	86 : 14
Cryptoxanthin (XXVIII)	1 -OH	82 : 18
Canthaxanthin (XL)	2 =O	50 : 50
Kéto-4 hydroxy-4' *-carotène	1 =O + 1 —OH	36 : 64
Isozéaxanthin (XXXVIII)	2 —OH (allyl)	22 : 78
Zéaxanthin (XXIX)	2 —OH	11 : 89
Astacin (XLIV)	4 =O	08 : 92

*Greek letter prefix apparently omitted from foreign original. Translator's note.

methylation, reduction, etc.) is very instructive [96]. But application of this technique has remained limited since it has been eclipsed by the success of analytic chromatography, paper or thin-layer and its use, in many research teams, is to indicate, among other characteristics, the partition coefficient in at least one system of solvents when a new carotenoid is being described.

We will note in passing that another criterion of the relative solubility of a compound is its behavior in partition chromatography. Thus, Egger [43], using as a stationary phase cellulose impregnated with oil (mineral or vegetable, according to the greater or lesser lipophily of the compounds studied), and as a moving phase a mixture, in suitable proportions, of acetone and acqueous methanol, observes that at absolute value 1 -OH# 1.5 C =O = 2 esters, hydroxyl and carbonyl evidently act in the opposite direction to the ester.

2.1.2 Relationships between adsorption and structure. The more a compound acquires, due to electrostatic attraction of a polar adsorbant, greater electronic asymetry, the more it is retained by the polar adsorbant. The oxygenated functions of xanthophylls play a leading role in the phenomenon, since oxygen tends to attract the electronic doublet of the carbon-oxygen bond; the order of increasing polarity is substantially that of the hydrophily [140]. The most polar radicals are the carboxyls and hydroxyls of an acid nature (α ketonic enol), then the hydroxyls, the carbonyls, and the epoxides. The influence of an alcohol is minimized by its substitution (either, ester). In thin-layer chromatography, the following quantitative relations could be established: 1 - OH # 1.5 C = O # 4-5 5-O - C - R (fatty acids /19

O

from C_{10} to C_{20}) [43]. As for the hydrocarbon skeleton, its adsorbability is stronger in proportion as the polyenic system is longer, since cyclization causes a decrease in polarity. The isolated double ethylene bonds increase the molecule's polarity, but to a variable degree according to their position; finally, a benzene cycle proves to be hardly more polar than if it carried only one conjugated double bond in the rest of the molecule [110]. Trans → cis isomerization can entail a change in polarity in either direction but a general relation cannot be established between this modification and that to the skeleton [36]. We should finally note that, expecially for the most polar of them, the order of relative adsorption of the carotenoids can vary slightly from one chromatography system to another [62], and a systematic study of these differences may reveal a source of structural information [93,140].

Table VIII

Chromotographic properties of various carotenoids on Schleicher & Schüll No. 287 paper (according to Jensen & Liaaen, Jensen [93] Arpin & Liaaen Jensen [9,10,11] Aasen and Liaaen Jensen [2].

Carotenoids	R_f VALUES				
	0*	2*	5*	10*	20*
β-carotène (XXVII)	0.95				
γ-carotène (XVI)	0.68				
Neurosporène (IV)	0.66				
Chlorobactène	0.62				
Lycopène (V)	0.53	0.68			
Cryptoxanthine (XXVIII)	0.29	0.62	0.81	0.91	
Torulène (XVII)	0.25				
Diester of plectaniaxanthine		0.47	0.80		
Chloroxanthine (IX)	0.13	0.46	0.73	0.90	
Déhydro-3,4 torulène		0.35			
Methyl ester of torularhodin			0.58	0.90	
Dehydro-2' plectaniaxanthin ester			0.57	0.90	
Dehydro-3,4 lycopène (VI)			0.52		
Spirilloxanthin (XII)		0.18	0.40	0.76	
Déhydro-2' plectaniaxanthin (XXII)			0.40	0.75	
Rhodopin			0.39	0.75	
Lutein			0.39	0.72	
Zeaxanthin (XXIX)		0.09	0.30	0.59	
Aldehyde of torularhodin (XXIV)			0.29		
Astaxanthin (XLIII)				0.57	
Plectaniaxanthin (XXI)				0.45	
Diester of phillipsiaxanthin				0.35	
Alcool of torularhodin (XXIII)			0.19		
Phillipsiaxanthin (XIV)					0.36
Torularhodin (XXV)				0.10	

(*) %Acetone in petroleum ether.

2.1.3 Relationships between visible UV absorption and structure. Absorption spectrometry in the visible range and the near UV, the first spectral method used—first in the simple form of spectroscopy—for characterization of many organic compounds, is constantly used in studying carotenoids. Among the numerous recent publications on this subject, or touching on it, we will cite those of Karrer and Jucker [94], Dale [33,34], Goodwin [62], Zechmeister [166], Weedon [159], Isler et al [87,88,89], Davies [36], Liaaen Jensen and Jensen [111] and, of course, Scott's general work [133].

2.1.3.1 Qualitative aspects

a) General absorption characteristics of aliphatic polyenes. The absorption spectrum of carotenoids essentially has three peaks in the visible range—or the near UV for the less unsaturated of them such as phytoene (I). The middle peak has the strongest optical density and serves to mark and compare the different pigments; of the two others, that with the longest wavelength is always the most clearly marked. The position of λ_{max} as the spec- /20
trum form is largely influenced by the nature of the solvent (Table IX); more generally used is petroleum ether. /

For a number of years, especially since the work of Karrer and Jucker [94] we have known the relationship between wavelength at the absorption peak and the number of conjugated double bonds of the polyenic system of carotenoids. The latter, like all polyenes, absorb longer wavelengths according as the number of conjugated double bonds is higher, and the substituant methyls each also account for a quarter of a double bond [133].

However, this increase of λ_{max} with the number of conjugated double bonds is not constant, as it decreases regularly by 25 to 7 nm per double bond when the initial number of these passes from 5 to 15 [111].

Correlatively, the λ_{max} of a carotene of unknown structure permits the number of double bonds making up its chromophore to be estimated, by comparison with the known values for reference compounds (Table X). In practice curves have been plotted [33, 159] from such values and the number of double bonds can thus be determined from the graph. However, since the spectrum increment decreases regularly when the number of double bonds increases, the curves become inflected and quickly make any interpolation inaccurate. We find that, by translating these data into semi-logarithmic coordinates, a much more convenient linear relation

TABLE IX

Principal absorptions (nm) of various carotenes in different solvents.

	Petroleum ether	Benzene	Chloroform	Carbon Sulfide	Reference
Lycopene	446 472 506	455 487 522	456 485 520	477 507 548	(90, 62)
γ-carotene	437 462 494	447 477 510	447 475 508	463 496 533	(90, 62)
β-carotene	425 451 482	466	466 497	450 485 520	(62)
ε-carotene	419 444 475	434 452 487	433 452 483	449 470 501	(90, 62)
δ-carotene	428 458 490	445 467 501	440 470 503	457 490 526	(62)

TABLE X

Effect of increasing the number of double bonds on the wavelength positions of the absorption peaks.

Compound	Number of conjugated double bonds	Absorbtion peaks (nm) (petroleum ether)	Reference
Phytoène (I)	3	276, 286, 298	(90)
Phytofluene (II)	5	331, 347, 366	(90)
ζ -caroténe (III)	7	380, 401, 425	(90)
Neurosporène (IV)	9	416, 440, 470	(90)
Lycopène (V)	11	443, 472, 504	(90)
Dehydro-3,4 lycopène (VI)	13	465, 494, 528	(105)
Bisdehydro-3,3',4,4' lycopène (VII)	15	480, 510, 540	(141)

is obtained (Fig. 4). This law can be shown by integrating the equation:

$$\frac{d\lambda}{dn} = \frac{K}{n} \quad \text{(where n = number of double bonds);}$$

$$\int d\lambda = K\int\frac{dn}{n} \quad \text{whence} \quad \lambda = \underline{a} \log.n + \underline{b}$$

In the present case the graphic method gives for a the value of 367 and for b the value of 90 nm; the other two peaks obey analogous laws. Such a logarithmic relation between the cause (electronic circulation in the double bonds) and the effect (maxi- /21
mum spectrum absorption) leads one to believe that oscillation of the chromophore is of the "self-damping" type.

However, the relation is strictly linear only for values of n between 3 and 13; above and below these values the curve inflects. It should be noted that an aliphatic carotene with C_{40} cannot contain more than 15 double bonds and the linearity maximum n = 13 corresponds to the end double bonds beginning to resonate, a phenomenon which must increase the damping of the system. The lower limit of the three double bonds corresponds to a non-substituted chromophore, entirely located between two methyls, which once more accents the influence of this group on conjugation of the polyenic system.

However, the spectrum pattern also varies with the number of double conjugated bonds: electronic spectra of aliphatic carotenes get sharper and sharper from the phytoene to the neuroporene, then regress clearly from dehydro-3, 4 lycopene.

b) Variations in fundamental absorption. The UV-visible properties of carotenoids vary a great deal, both in the λ_{max} and in the shape of the spectrum, as a function of the following broad types of factors:

- nature of the end of the polyenic chain;
- presence of conjugated carbonyl groups with the polyenic chromphore;
- the cis-trans configuration.

(i) The nature of the ends of the molecule. At the end of the type β-carotene, the double bond at 5-6 inside the terminal cycle causes the chromophore to lengthen to one-third of the contribution of the corresponding double bond of an aliphatic carotene [109] (Table XI), which corresponds to a decrease in its conjugation with the polyenic chain.

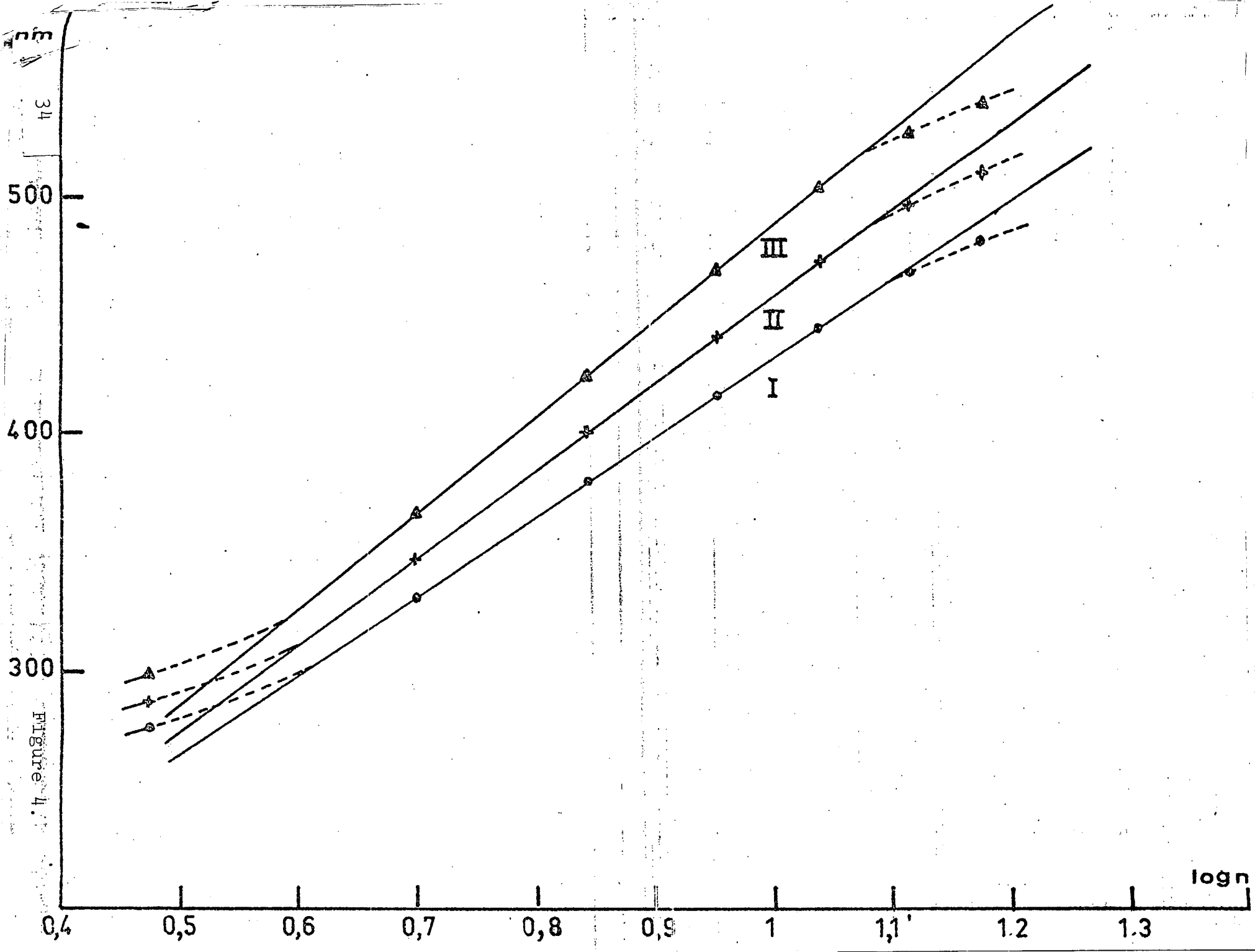


Figure 4

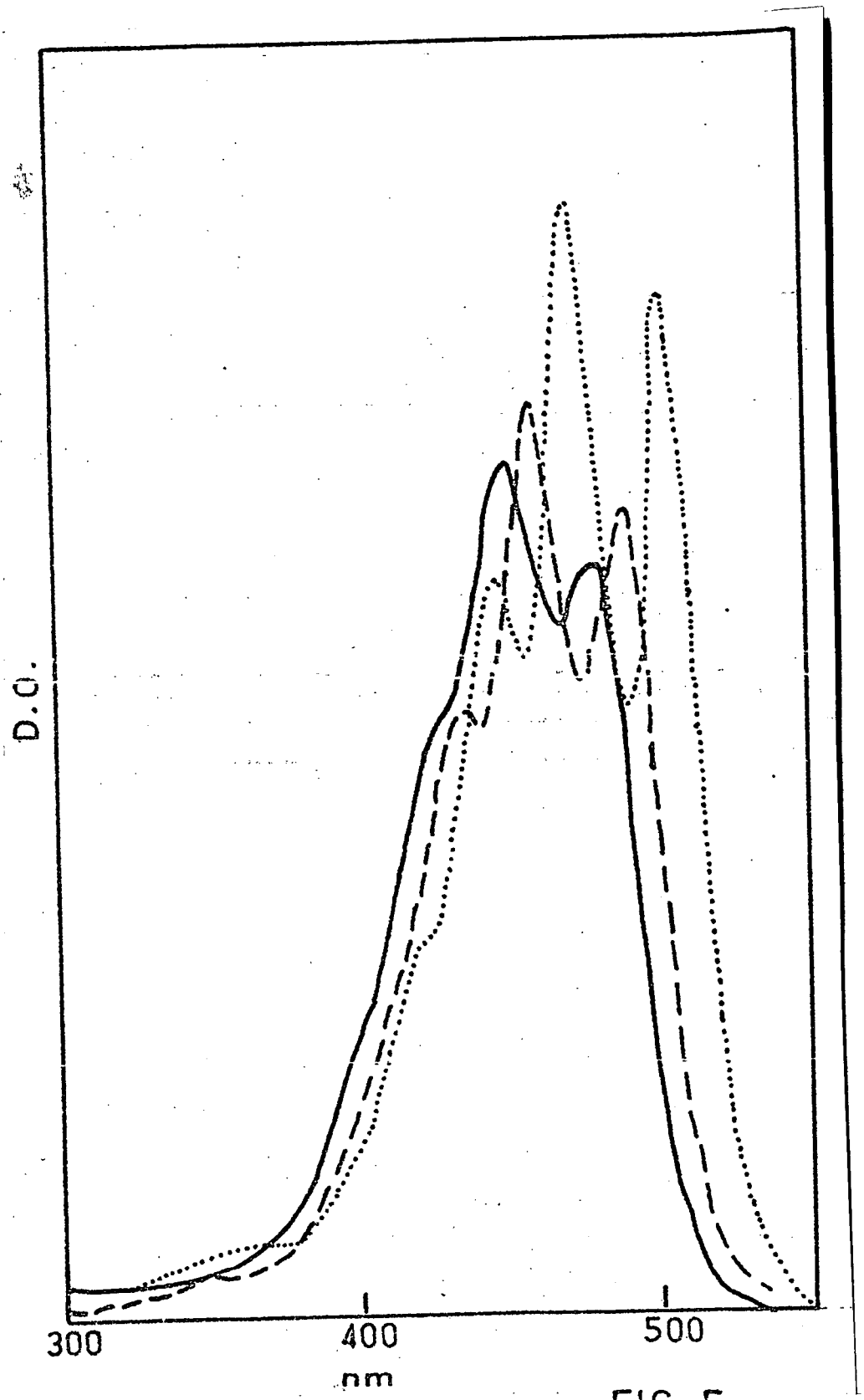


FIG: 5.

TABLE XI

Various bathochrome effects of carbonyls groups.

Compounds	Principal absorption (nm. petroleum ether) or hexane(*)	Spectrum shift	Reference
Spheroïdene	455	28	(105-106)
Spheroïdénone	483		
Spirilloxanthin	494	24	(103-104)
P. 518	518		
Plectaniaxanthin	474	29	(6)
Déhydro-2' plectaniaxanthine	503		
Torulene	484	5,5	(90-2)
Céto-4 torulène	489,5		
Retro-déhydro-β-carotene	472	11,5	(90-90)
Keto-3-retro déhydro-β-carotene	483,5		
β-carotène	450	8	(94-36)
Echinénone	458		
Canthaxanthin	465	7	(90)

* Solvent : hexane

Like the wavelength, the fine structure of the spectrum decreases with cyclization: the spectral pattern, characterized by three clearly marked peaks in the aliphatic carotenoids, becomes progressively more blurred as we pass to the monocyclic then bi- /22
cyclic carotenoids. Liaaen Jensen [103] chose the ratio of the relative height of the peak of the longest wavelength (III) to the middle peak (II) as characteristic of this pattern: it is 0.80 for lycopene (V), 0.60 for γ-carotene (XVI), and 0.33 for β-carotene (XXVII) (trans forms). Correlatively the peak of the shortest wavelength blurs out and is represented only in the shape of a shoulder in the case of β-carotene. Moreover, the relative value of the optical densities of the absorption trough between peaks II and III and of peak I also varies according to the nature of the skeleton: the first is smaller for aliphatic carotenoids, and larger for bicyclic carotenoids. Monocyclic carotenoids have an intermediate position variable according to the relative size of the aliphatic and cyclic chromophore portions.

Of course, cyclization only affects the spectrum it if concerns the chromophore: ε-carotene, whose two extreme double bonds only included in the terminal cycles are not conjugated to the remainder of the molecule, has a spectrum comparable to that of the neurosporene, whose chromophore it possesses.

The absorption of retro-carotenoids (in which the whole system of conjugated double bonds has had one carbon displaced, such that the central chain is attached to the ends by a double bond) for one and the same number of conjugated double bonds, is placed between that of the normal cyclic carotenoids and the absorption of the aliphatics: their double bond in the cycle is more conjugated than that of β-carotene.

The contribution of the aromatic ends of certain carotenoids (bacterial) to the molecule's chromophore varies with their substitution; in particular, an end of the trimethyl-1,2,5 phenyl type has exactly the same effect as a terminal cycle of β-carotene [109].

(ii) Introduction of oxygenated groups. This acts qualitatively on the spectrum if it causes a double conjugated bond to disappear (by hydration leading to hydroxyl; also epoxide and furanoid groups) or appear (carbonyl) on the chormophore.

Conjugation of a carbonyl group with the polyenic system /23
produces three striking effects: clear decrease in the fine structure of the spectrum, bathochrome shift, hypochrome effect. The first two are illustrated by Figures 6 and 7 and are explained

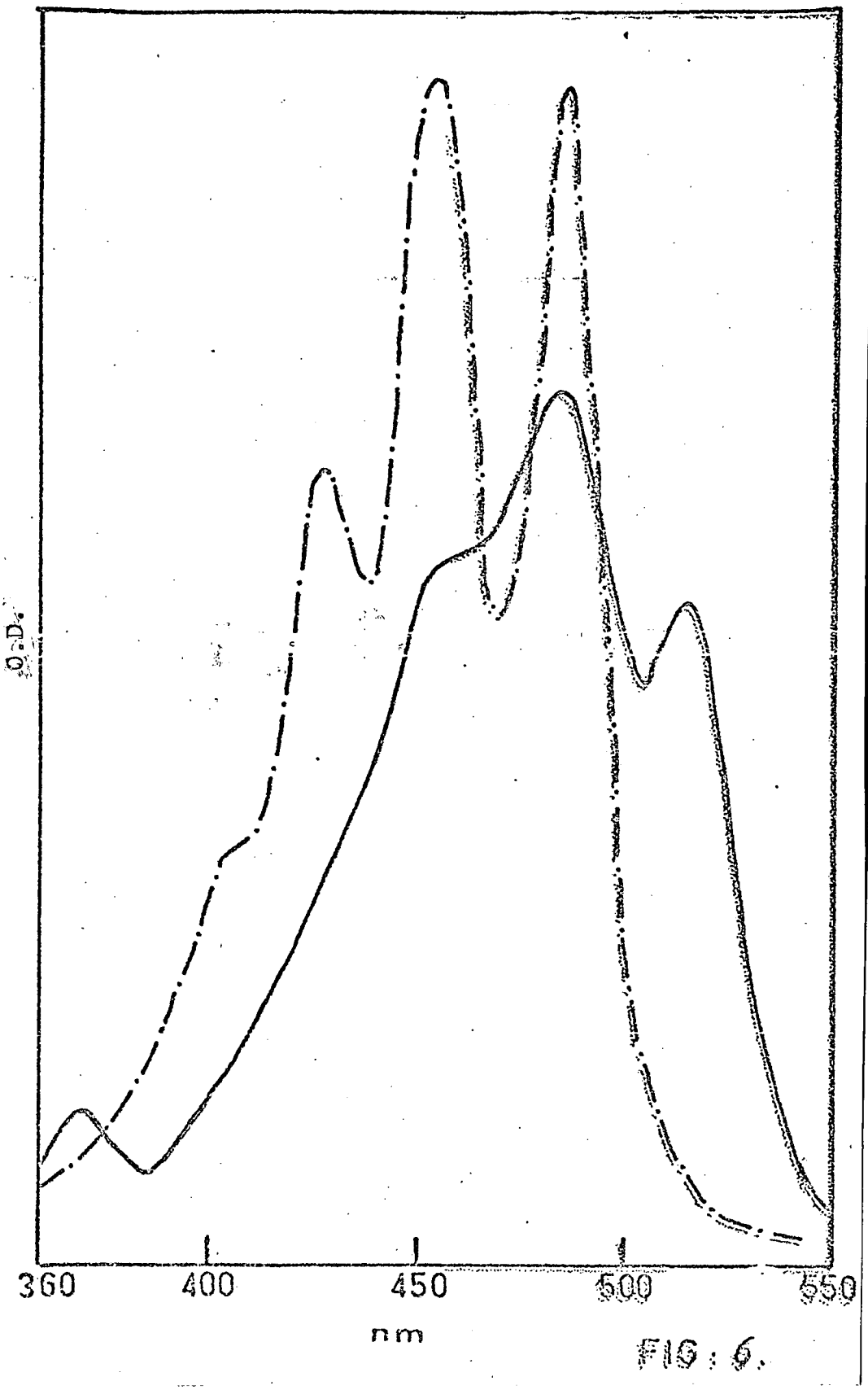


FIG : 6.

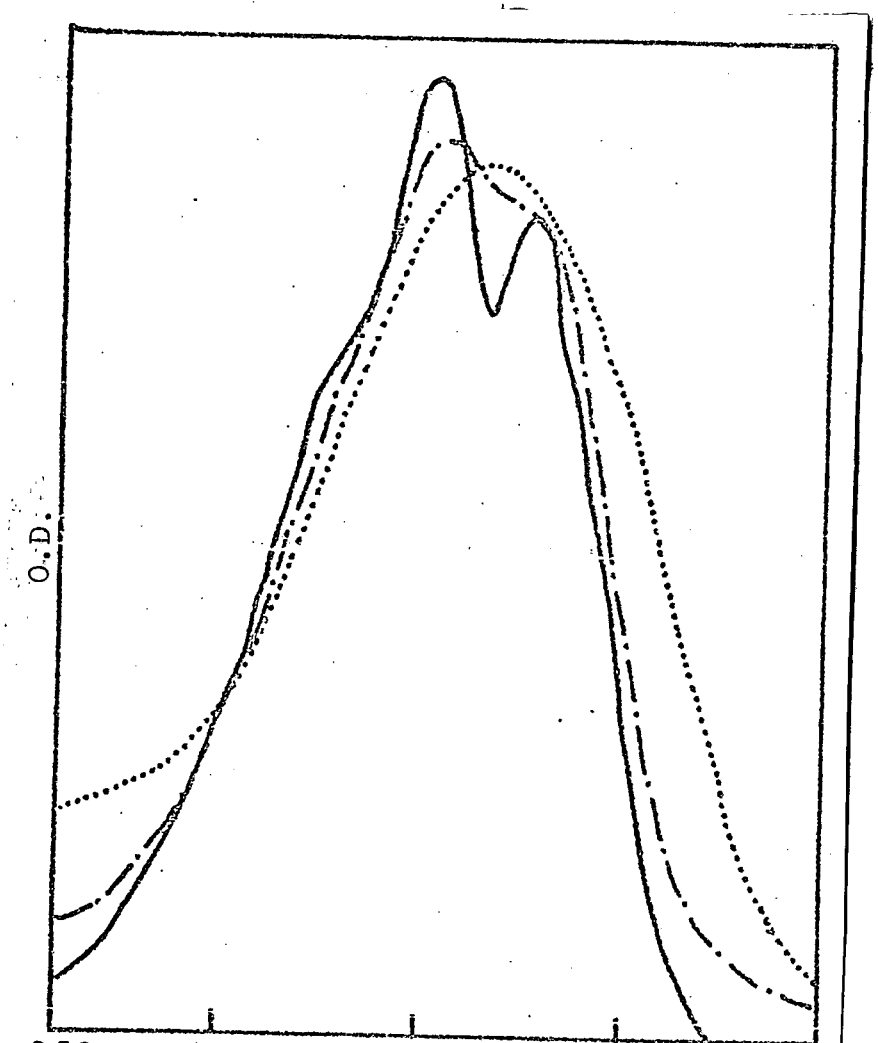


FIG: 7.

below; the third effect, quantitative, will be illustrated further in the text.

We observe very different bathochrome effects according to whether a carbonyl group is introduced on the cyclic or aliphatic ends: compared to the bathochrome shift observed in aliphatic carotenoids (which can be as much as 28 nm), that of the cyclics is much smaller (7-8 nm); the keto-retro-carotenoids have an intermediate position here also (Table XI).

Presence of a second conjugated carbonyl group at the other end of the molecule (carbonyls in position ω-ω') is expressed very differently in an open-chain carotenoid and in a bicyclic carotenoid (111). In the first case this second carbonyl has only a slight bathochrome effect: we observe only 24 nm difference between the spirilloxanthin and its di-substituted homolog, P. 518, which led to the belief that the latter was only a monoketocarotenoid [104]. For the cyclics, on the other hand, the second carbonyl has a bathochrome effect equal to the first (see the λ_{max} d β-carotene, echinenone and canthaxantin).

In the very special case of neighboring ketone groups (as in astacin, positions 3, 4) there is no substantial difference between the diketonic derivative and the monosubstituted derivative where the carbonyl (at 4 here) is conjugated with the chromophore.

A carbonyl group can come in different forms: ketone, aldehyde, acid, or ester. Liaaen Jensen [111] showed that for conjugated carboxylic esters the bathochrome shift is more limited than for the other carbonyls. Thus, the absorption peaks of torularhodin and its aldehyde are located at 407 nm, while the methyl ester of torularhodin absorbs at 497 only [11,90,89]; the shift is thus only 13 nm as compared to torulene, while it is 23 for the acid.

(iii) The cis-trans configuration of carotenoids. Thus far /24
we have only looked at the problem of carotenoids in which all the double bonds were in the trans form, which is the general case for natural products. Various factors (light, temperature, oxygen, iodine) trigger isomerization of one or more double bonds from the trans to the cis form. Zechmeister [166] made an extremely detailed study of the spectrum characteristics of these cis isomers. The electronic spectrum of a cis carotenoid is differentiated from that of the entirely trans compound by several features, both in the visible and in the UV ranges. We will summarize these:

1. The absorption peaks shift towards the shorter wavelengths.

2. The fine structure of the spectrum decreases clearly. Liaaen Jensen [103] defined the ratios permitting the greater or lesser isomerization of an originally trans compound to be determined.

3. But the main feature of cis isomer is the appearance in the near-UV (at about -140 nm of the peak of the longest wavelength) of a peak which can double in the case of aliphatic carotenes.

Note: It will be remembered that in the near-UV other substances than colorless carotenes, such as various steroids, absorb other lipophile substances. Thus, in the fungi, ergosterol is very often encountered (two double bonds conjugated at 5.6 and 7.8) whose absorption spectrum in the UV has a certain relationship (λ_M(260) 270, 282, and 294 nm) with that of phytoene (I) (λ_M 276, 286, and 298 nm.

2.1.3.2 Quantitative aspect. For a given thickness of solution traversed and given concentration, at each wavelength and in particular at maximum optical density, light absorption by a compound is a function of its type, which may be expressed by its coefficients of specific or molar extinction. The first is expressed in $E^{1\%}_{1cm}$ values (optical density corresponding to absorption of 1 cm thickness of a solution of 10 mg substance dissolved in 1 cm^3 of solvent), the second is symbolized ε ($\varepsilon = E^{1\%}_{1cm} \times \frac{M}{10}$, M being the molecular mass of the compound). /25

For a given solvent and wavelength, the value of this coefficient is a constant of the product and measuring it permits:

- its purity to be tested;
- indications to be obtained on its very nature, since the value of this coefficient varies a great deal from one carotenoid to another, as shown in Table XII. We se that:
  - in the series of aliphatic carotenoids, absorption increases regularly from the phytoene to the lycopene (the latter having the greatest value known for carotenoids);
  - passage of aliphatic carotenoids to homologous bicyclics is accompanied by a sharp drop in absorption, as shown by comparing the values for lycopene, γ-carotene and β-carotene;
  - introduction of a carbonyl group as a very substantial hypochrome effect: from β-carotene to echinenone, absorption decreases by 20%;
  - the retro systems here have an absorption intermediate between those of cyclic and normal aliphatic carotenoids, both for hydrocarbons and for carbonylated compounds.

TABLE XII

Specific extinction coefficients of various carotenoids

Caroténoïds	ε	λ_{Max}	References
Phytoène	49,800	286(EP)	(39)
Phytofluène	85,500	347(EP)	(39)
ζ -carotène	138,000	400(EP)	(39)
Neurosporène	155,000	440(EP)	(39)
Lycopène	185,500	472(EP)	(111)
γ -carotène	166,200	462(EP)	(111)
β -carotène	137,200	451(EP)	(111)
α -carotène	150,000	444(EP)	(90)
ε -carotène	154,900	444(EP)	(90)
Rétro-déhydro- β -carotène	171,800	472(EP)	(90)
Kéto-3 retro-déhydro- β -carotène	142,500	483,5(H)	(48)
Echinénone	120,800	458(EP)	(36)
Canthaxanthine	111,000	468(H)	(47)
Cryptoxanthine	135,500	451(H)	(62)
Zéaxanthine	133,000	451(H)	(62)
Torularhodine	115,000	507(EP)	(90)
Eschscholtzanthine	185,600	464(H)	(90)
Rhodoxanthine	140,200	490(H)	(48)

H : hexane.
EP : petroleum ether.

Conversely, specific absorption of a carotenoid permits it to be assayed: if at its λ_{max}, a solution with volume V cm^3 of a compound with coefficient $E^{1\%}_{1cm}$ has an optical density OD (measured on a thickness traversed of 1 cm) the weight W of the pigment, expressed in mg, will be given by:

$$W = \frac{OD \times V \times 10}{E^{1\%}_{1cm}}$$

2.2 Analytic Process

/26

All the operations described below are done in darkness or diffused light. The isolated fractions are kept under nitrogen in the freezer. The chemical reactions which are not immediate are generally carried out under nitrogen, with temperatures individual to each case.

2.2.1 Extraction. The biological material, dehydrated by standing in acetone, is treated by the same solvent at low temperatures (by crushing in a mortar for the small specimens with the aid of a special mixer for the larger ones) which is renewed until a colorless extract is obtained. Generally this extraction is easy to do, since most of the carotenoids are brought out into the pure acetone when the material is ground. In some cases, however, this solvent can be advantageously reinforced by enrichment with 20% methanol, and the ground material can be completely extracted by leaving it overnight in the methanol in the refrigerator. The extraction residue is kept for its dry weight to be measured.

Whatever extraction procedure is used, the solutions obtained are added to petroleum ether (boiling point 40-60° C) and the quantity of a water necessary for the two phases to separate: after stirring and decanting, the carotenoids are found in the petroleum ether upper phase. It may be necessary to recommence this operation to get all the carotenoids, but frequently the hydro-acetone hypophase is colored by pigments of different kinds.

The ether-petroleum solution is rinsed several times in water (pure, or first containing 20% sodium chloride or ammonium sulfate if too large emulsions tend to appear) to eliminate all traces of acetone, then dried on anhydrous sodium sulfate. Its absorption spectrum in the visible and UV ranges is then quantitatively recorded (Beckman DB recorder spectrophotometer). Aside from the qualitative indications given in many cases, the total quantity of

pigment present in the extract can be determined (taking as a basis of calculation the specific absorption of the pigment which analyses show to be very much preponderant, or a mean value interpolated according to the composition of the mixture) and finally /27 shows up the sterols which often accompany them.

This solution is then concentrated to a small volume under low pressure with a rotating evaporator at a temperature not above 40° C, and left overnight in the freezer; if they are fairly abundant, the sterols largely precipitate and are then eliminated by filtration or centrifugation.

2.2.2 Saponification. This operation must be avoided where possible, or at least retarded, since it often causes partial alteration (by stereoisomerization or oxidation) of certain carotenoids; if performed on the crude extract, it prevents us from ascertaining the form in which the xanthophylls it contains are present in nature. It is, however, necessary—aside of course from showing up the ester functions of certain carotenoids—whenever the chromatographs are disturbed by overly large quantities of lipid impurities.

Saponification is done with potassium (usually 5% KOH in the ether ethyl-methanol mixture 1:3 by volume), at ambient temperature, usually for four hours [103]. We then add water until demixing. The carotenoids will be in the epiphase. The first hypophase is later extracted again with ether; if it contains pigments which are then clearly hypophasic (acid) these are collected in a new etherated fraction after adding acetic acid, and are thus isolated [49].

2.2.3 Fractionation. This is done essentially by adsorption chromatography. It was for a long time usual, before performing this operation, to divide the carotenoids (according to their solubility) into epipigments and hypophasic pigments [36]. The great flexibility of preparative chromatography renders this first step superfluous except where the total extract contains an extremely polar xanthophyll—and correlatively a rather hypophasic one—the normal adsorbants of which are difficult to elute quantitatively: its very singular solubility then enables it to be easily extracted. Thus, the acid pigments (torularhodin, astacin) are advantageously separated from the initial mixture before any /28 chromatography by extraction with acqueous alkaline methanol.

Various adsorbants have been considered for carotenoid chromatography [94,62,36,140]. The one most commonly used today is alumina, more or less deactivated (according to Brockman and

Schodder's standardization) by hydration, according to the polarity of the molecules to be separated, degree of activity II is the most common; handling is then easy since magnesium, which perhaps gives a somewhat finer separation [36] comes in an almost colloidal form, requiring addition of diatomaceous earth which facilitates filtration.

The chromatography tube, set up to be truly vertical, size according to the quantity of substances to be separated, is filled with petroleum ether. Above a light fiberglass stopper, the alumina is poured very slowly and regularly, such that all the air escapes and it settles as homogeneously as possible. Alumina can also be first placed in suspension in petroleum ether in a decanter bulb (it is thus easier to ensure that the air is expelled) and the paste obtained when left to stand is made to flow into the chromatography tube. In every case, it is well to tap the apparatus during this preparation to ensure that the adsorbant is settling regularly. When there is a good heap of this, it is topped with a 1-2 cm thick layer of a powder with no adsorbing properties (seashore sand, for example) such that the surface of the alumina is protected from any eddies when the solution to be analyzed or the solvent is poured in, and to ensure a perfectly regular initial front when chromatography begins.

Brought to low volume (but enough not to risk crystallization or precipitation of one or other of the solutes) the ether-petroleum solution is placed at the top of the adsorbing column, then petroleum ether is poured in ensuring percolation of the initial solution through the alumina; the various carotenoids, more or less retained, separate in the form of differently colored bands or zones. The chromatogram is thus developed and the various fractions are eluted in turn by progressive increase of the "force" of the moving phase: petroleum ether gradually enriched with ethyl /29
ether, then acetone; then as there is a risk of the latter solvent dehydrating the alumina and thus increasing its adsorption force, benzene with, if necessary, a growing proportion of methanol is added. For extremely polar pigments, it may be necessary to add acetic acid or alkaline methanol. Due to the coloration of the compounds studied, the behavior of the various fractions is easily observed, permitting the polarity of the moving phase to be increased as chromatography develops, in successive increments, which is an easier and more flexible way of operating than using an elution gradient [36]; thus, it is fairly simple to collect the different fractions separately as they come out of the column, isolating intermediaries if need be when two bands overlap. An automatic fraction collector, much more rigid is of little use here.

One can also extrude the different zones separately when, since elution is too slow, the risk of the pigments altering upon contact with alumina is a more important consideration than the progress of their separation which more prolonged chromatography would involve.

If the solvent flow must be speeded up, it is preferable to do so with the aid of overpressure in the upper part of the chromatography tube (using a column of solvent, or nitrogen under pressure) than to aspirate the lower part, since the latter breaks up the homogeneity of the adsorbant.

2.2.4 Characterization of isolated fractions

2.2.4.1 Spectrophotometry. The UV-visible spectrum of each fraction so isolated enables its purity to be assessed to some extent by comparison with that of the reference products, or more generally the mixtures to be detected according to the anomalies in their spectral patterns. But, above all, if the compound has otherwise been shown to be pure, its spectrum, as we have seen, gives rich information on its structure from the qualitative viewpoint and, if it crosschecks the chromatographic behavior, provides an extremely valuable index for identification; quantitatively, the carotenoid content present is determined, as we said above, by taking as specific absorption that of the pigment to which the fraction had been identified (or failing this, a value interpolated /30 according to the assumed structure, or a mean arbitrary value, generally $E^{1\%}_{1cm} = 2500$). The quantities so calculated give the relative importance of the various carotenoids; the absolute contents are obtained by comparing these proportions to total extract adsorption, due to losses and retentions during the analysis.

2.2.4.2 Analytic chromatography. This permits the purity of a fraction to be established and its polarity to be determined fairly accurately, and giving more accurate information than preparative chromatography; finally, if we have a reference produce* to compare it to the natural compound by chromatography. For this

*We would like to thank Dr. K. Egger (Privat-Dozent at the Botanical Institute of the University of Heidelberg) and Drs. O. Isler and H. Thommen (Ets. Hoffmann - La Roche, Bale) who procured for us samples of reference carotenoids, in particular ketone derivatives of β-carotene.

we used two different chromatography systems:

a) Silica gel in the form of a thin layer, according to the process described in detail in the French translation of Randerhart's book [192]. The adsorbant used is G Merck silica gel, the solvent varying with the polarity of the fraction studied and the purpose sought: the least polar carotenes are separated by petroleum ether (coiling point 90-110° C) [36], by petroleum ether (boiling point 40-60° C) enriched with 2% acetone [132], or by methylene undecane-chloride solvent (80-120) [139], the xanthophylls by methylene chloride containing 20% ethyl acetate [139] or by petroleum ether (boiling point 40-60° C) with 20% acetone added [43], while all the carotenoids migrate and separate with the benzene-ether ethyl-methanol mixture as a moving phase (85-10-5) [144]; the latter system is thus particularly interesting for preliminary "exploration" of a natural extract.

b) Filter paper enriched with mineral adsorbant. Although /31
the idea is already quite an old one, it has been widely applied only in the last decade [93]. This technique, from Jensen's Norwegian school [93,103,92], is characterized by an incomparably high resolving power, exceptional facility and rapidity of execution, and quantitative reproducibility and yield rarely attained in this domain. Since it is little known, although extremely fruitful, we will describe it in some detail.

The method of development chosen was circular chromatography according to Rutter [131], which method, other things being equal, gives the best resolution [92]; indeed, the solvent diffuses simultaneously not only in the direction of migration of the substances to be separated, but also tangentially: the zones which become individualized are thus stretched transversally and do not overlap, even when the difference in their behavior is minimal. Such a mode of migration obviously restricts the chromatogram to a single dimension, but the two-dimensional technique, longer and requiring an intermediate drying phase, is of little use in the case of such unstable pigments as carotenoids [92,36].

Discs of filter paper, e.g. 18 cm in diameter, are used. A radial strip about 2 mm wide is cut out almost to the center; its length is brought to about 2 cm, it is inclined perpendicular to the surface of the paper, and will be used to feed the chromatogram with solvent. The solution for analysis is placed drop by drop in the center of the disc where a nitrogen stream ensures that the solvent evaporates quickly; the spot, as small as possible, must not exceed 1 cm diameter. After deposition, several drops of acetone, applied to contact the central end of the strip,

cause it to shape a narrow arc of a circle: the zones which separate on development are thus finer and more regular. For chromatography, the useful part of the paper disc is divided into three equal parts by additional radial incisions, stopping a few mm from the center; the substance to be studied is deposited, as before, at the top of one of the lateral sectors thus defined, the control compound of the other side and the two, in a mixture, in the center portion. /32

The paper disc is then placed between two identical lids of a Petri dish, the lower one filled almost three-quarters full with solvent into which the above strip is plunged. Development, done in the dark, is achieved in 10-20 minutes. It may be lengthened for separation of low R_f pigments, when the solvent evaporates by the surface of the paper outside the Petri dish (lost front chromatography).

By careful handling and using fresh solvent for each chromatography, the R_f values are reproducible within ±0.01 [93], provided however that development is not disturbed by overly large quantities of lipid impurities, and for a given batch of paper.

In preparation, the various bands are cut immediately after separation to reduce pigment alteration to a minimum, they are heaped up in the bottom of fine glass tubes tapered at one end, and eluted by acetone: the pigments deposited are recuperated with a yield reaching 98-100% [93].

The adsorbant-eluant system used is, for all pigments with polarity greater than or equal to that of β-carotene, Schleicher and Schüll paper No. 287, containing 20% Kieselguhr, the moving phase being petroleum ether (boiling point 40-60° C) possibly enriched with a percentage of acetone proportional to the polarity of the pigments to be separated [93]. Filter paper containing alumina (S & S No. 667 or 288), slightly basic and adsorbing more strongly, was used successfully for separation of carotenoids with polarity between β-carotene and phytofluene [93]; it could also be applied to the study of more polar carotenoids, increasing the eluant power of the moving phase, the separations obtained being somewhat better, in particular for the hydroxylated isomers, than with Kieselguhr paper [93]. This, however, much less difficult to handle, remains the usual adsorbant, since the two systems fruitfully complement each other for cochromatography comparisons.

The resolving power of the system is remarkable, as shown by /33 the excellent separation of the various stereoisomers of one and

the same pigment [93]. Thus, although it is well fitted for this, it is less used for separation of a complex mixture of carotenoids in large groups of the same polarity [93] than for very fine analysis, both quantitative and qualitative, of a fraction of given polarity, the kinetic study of a characteristic reaction [93,9], accurate determination of the polarity of a compound, all roles in which presently it is irreplaceable. In the case of very small specimens, analytic chromatography of the overall extract, if possible before and after saponification, possibly in the presence of reference substances, gives semi-quantitative information and, as we shall see, often conclusive information from the qualitative viewpoint, at least for the major pigments.

2.2.4.3 Partition coefficient. We looked at the contribution of this measurement to structural elucidation of a carotenoid above.

2.2.4.4 Characteristic reactions. In the field of carotenoid chemistry these have developed over the last decade in parallel to spectral techniques since not only can they confirm and even complete such information, but they are able to furnish almost conclusive results (due to the resolving power of chromatography and spectrophotometry) without having anything like the stringent requirements for quantity and purity, We will confine ourselves to discussing those we have had occasion to use directly.

a) Acetylation, presently done by acetic anhydride in a pyridine medium, substitutes only the primary and secondary hydroxyls. Their number can be determined by the difference in polarity or hydrophily between natural xanthophyll and its peracetylated derivative, or by showing up the various intermediates of the reaction, with periodic chromatographic analysis of aliquots of the reaction medium.

b) Silylation [114] is also positive with tertiary hydroxyls,/34 giving a positive index for their presence, which could hitherto only be shown negatively by the hydrophily residue of the acetylation product of natural carotenoid.

c) Reduction by lithium-aluminum hydride [105] of the carbonyl groups of carotenoids gives a more polar compound whose visible spectrum is that of the parent carotene. This reaction also opens the door to hydroxyl characterization reactions described above.

2.2.4.5 Determination. It is reasonable, when ascertaining the structure of a carotenoid, to take as a working hypothesis

that the molecule possesses the classical skeleton with C_{40}, pigments of this family, although a number of natural exceptions are known: apocarotenoids, and bacterial C_{50} carotenoids. Partition chromatography according to Egger (thin layer of cellulose impregnated with triglyceride/acetone and acqueous methanol) [42], very sensitive to the length of the hydrocarbonated chain of the molecule, would later enable this to be ascertained.

The absorption spectrum in the visible range supplies, as we have seen, very accurate indications on the chromophore. In the case of a carbonylated compound, the spectrum of the product of reduction indicates the polyenic system present, the hypsochrome effect on reduction gives an idea of the number of carbonyls which have reacted (which will give the variation in polarity and hydrophily). The chromatographic behavior (on the preparation column then, more precisely, in an analytic process) indicates the polarity of the molecule, the partition coefficient, and its hydrophily. Comparison of these results to those given by the spectrum permits a conclusion as to the presence of hydroxyl groups (or carboxyl groups, recognizable from the acidity of the molecule carrying them) and permits knowledge of how many there are. Confirmation is obtained by acetylation then, if necessary, silylation.

In the case of already-known pigments, the above information alone leads easily to their identification. It is quite evident, however, that the most decisive criterion is direct comparison /35
with the reference compound, by thin-layer cochromatography or paper cochromatography using the thrice-divided disc technique, as much as possible in several different systems. To increase the significant value of such a comparison still further, it is desirable to compare not only the compounds themselves but the mixtures of stereoisomers to which they give rise under identical conditions, and all the homologous products which both furnish during this or that characteristic reaction.

CHAPTER III

EXPERIMENTAL RESULTS

/37

1. ANASCOSPOROUS YEASTS

Sporidiobolus johnsonii Nyland

The pigments are extracted by acetone then by methanol; the ether-petroleum solution is concentrated and put in the freezer: the sterols precipitate and are eliminated by filtration. The total carotenoid content amounts to 0.014% of dry weight [49].

The ether-petroleum solution is compared to 90% methanol containing 5% postassium which separates any acid pigments which may be present (hypophasic) from the carotenes proper (epiphasic).

After saponification, the epiphase pigments are separated by column chromatography, alumina II, into three components: β-carotene, γ-carotene, and toruelene, the purity and identify of the fractions being determined by spectrophotometry and (co-)-chromatographies on Schleicher & Schüll Nos. 287 and 288 paper.

The Sp. johnsonii hypophase proves to be perfectly identical to that of Rhodotorula sanniei, both by the absorption spectrum in the visible range (corresponding to a mixture of stereoisomers of torularhodin) which, by Schleicher & Schüll No. 287 paper cochromatography (solvent: 10-20% acetone in petroleum ether) and on thin layer with silica G gel (solvent: 2% methanol in benezne).

In total, the proportions of pigments are established as follows:

0.014%	β-carotene	: 31%
	γ-carotene	: 6%
	Torulene	: 20%
	Torularhodin	: 43%

Rhodotorula sanniei (Cif. and Red.) Lodder

This strain was analyzed by Tchang [143] and cultivated by us to provide pigment controls for red yeasts: torulene and torularhodin.

/38

Under our conditions of culture, the total carotenoid content amounted to 0.047% of dry weight, the various pigments being distributed as follows:

0.047%	β-carotene:	16.6%
	γ-carotene:	4.2%
	Torulene:	44 %
	Torularhodin:	35.2%

It should be noted that Tchang, although with cultures in a gel medium (malt dust plus 10% saccharose) obtained a total carotenoid content of 2.9% dry weight, torularhodin representing 99.4% of pigmentation. Faced with these values, we might ask (as Goodwin implied [59]) whether an error of 10 slipped into quantitative determination of the latter pigment; if we make such a correction his figures both for the total carotenoid concentration and the torularhodin content are still very much higher than ours. These differences might be partially explained either by development of the strain in multiple replantings over the last three decades separating the two analyses, or by differences in the development medium.

Rhodotorula glutinis (Fres.) Harrison

The strain studied (LY 422) was isolated by Professor Boidin; we collected all the assimilation tubes, the cultures in liquid and solid media, etc. used for its determination [20], the whole giving under one gram of dry material.

The overall spectrum [459, 487, 530 nm, and in UV the ergosterol peaks with O.D. 282 / O.D. 487 = 6) indicates, taking specific absorption of torularhodin as a basis for calculation, pigment content of about 0.36% of dry weight.

We are reporting this analysis only to indicate that, after fractions assimilated according to their chromatographic and spectrum behaviors respectively with β-carotene and γ-carotene, and before torulene and torularhodin (major) a pink fraction was eluted (by 10% ethyl ether, alumina II, spectrum 460, 419, 528 nm. /39
The study of this was not pursued, but results later obtained on other species led us to think that this may be a partially isomerized torularhodin ester.

0.36%	β-carotene	: 12%
	γ-carotene	: 7%
	Torulene	: 3%
	Torularhodin	: 68%
	"P 419" (ester ?) approximately	10%

Rhodotorula aurantiaca (Saito) Lodder

After Peterson et al [126] and Hasegawa et al [72] noted that, if carogenogenic yeasts as a whole could subdivide according to the wavelength of the absorption peak in the visible range of the ether-petroleum solution they supply, in Flavotorula (yellow, γ_{Max} 450 nm) and Rubrotorula (red or pink, γ_{Max} 480 nm), Rhodotorula aurantiaca - as one might suspect from its orangy color - is particularized by an absorption peak at 470 nm. Peterson et al [126] found that this species (cultivated in an agitated liquid synthetic medium) accumulated carotenoids at the rate of 0.011% of its dry weight; side by side with β-carotene (6.2% of total carotenoids) and γ-carotene (9%), 84.8% of its pigmentation is due to a compound which they believed to be close to torulene, but differing a little in spectral properties and greater solubility in methanol.

Eight liters of culture medium furnished 6.35 g of yeasts (dry weight). The epiphase obtained by extraction presents a fairly blurred spectra with peaks at 444, 469, and 500 nm. On the basis $E^{1\%}_{1cm}$ at 469 nm = 3000, a total carotenoid content of 0.021% of dry weight was calculated.

Upon analysis (after energentic saponification required by the presence of very large quantities of lipids), besides the low-content β and γ-carotene, we find, eluted from the alumina I column by 10% ethyl ether in petroleum ether a pigment whose spectrum in hexane has a pattern recalling a monocyclic carotenoid with peaks at 448, 475, and 501 nm. Its partition coefficient /40 between petroleum ether and methanol at 95% is 99:1. In view of these data, it is difficult to advance any hypothesis as to the structure of this compound; its spectrum appears to be intermediate between those of γ-carotene and torulene. We have seen that it was isolated after saponification: it seems not to be an ester; according to the data of Petracek and Zechmeister [127] its partition coefficient could be that of a retro-carotene or an

ether. We will finally report that lithium-aluminum hydride causes no modification to the spectrum of this compound:

	β- and γ-carotene	:	traces
0.021%	"P 475"	:	major

2. HETEROBASIDIOMYCETES

2.1 Ustilaginales

Ustilago scabiosae (Sow.) Wint.

The strain studied came from the Centraalbureau voor Schimmelcultures (CBS 177 - 42) and was kept in the laboratory under reference number LY 5815. 81 in the culture medium furnished 14.4 g of microorganisms (dry weight). The pigments extracted by acetone are shown to be epiphasic; the spectrum of the ether-petroleum solution thus obtained is very similar to that of β-carotene. Calculated from the absorption coefficient of the latter, the U. scabiosae carotenoid content, under our cultural conditions, was 0.0094% of dry weight.

Alumina II column chromatography enables separation of:

- a very large yellow-orange fraction, eluted by petroleum ether. Its spectrum is that of β-carotene, from which it cannot be separated on S&S 287 and 288 paper cochromatographies. This fraction represents 88% of the total of carotenoids present.

- two extremely close orangy bands, eluted together by 5-10% ethyl ether in petroleum ether. The spectrum and the cochromatography behavior (on S&S 287 paper) with control γ-carotene lead to the belief that the latter pigment is involved, /41
partially isomerized, and representing some 7% of the pigmentation.

- four yellow or yellow-orange bands, extremely minor, the small size of which did not permit further study:

	β-carotene	:	88%
0.0094%	γ-carotene	:	7%
	4 pigments	:	5%

Ustilago zeae (D.C.) Cda.
(CBS 44563 = LY 5824)

11.03 g of the biological material (dry weight) were entirely extracted by acetone; the ether-petroleum solution obtained presents, in the visible range, the spectrum of β-carotene; on the basis of specific absorption of this, the total carotenoid content amounts here to 0.0034% of dry weight.

After saponification, analytic chromatography on S&S 287 paper using petroleum ether as a moving phase (boiling point 40-60°C) shows a very major yellow-orange band (R_f 0.92), a orangy band (R_f 0.76: γ-carotene?) and a slight pink band (R_f 0.57: lycopene?). On the column first a very large yellow-orange fraction is eluted by petroleum ether, then a yellow band and a pink band displaced by the petroleum ether mixture: ethyl ether 1:1; these fractions, too small in content, could not be studied.

0.0039%	β-carotene	90%
	γ-carotene	?
	Lycopene	? traces
	1 pigment?	

2.2 Auriculariales

Auricularia auriculajudae Lin. ex Fr.

The epiphase spectrum obtained reveals only a very slight chlorophyll contamination of the specimen.

2.3 Tremellales

Guepinia helvelloides D. C. ex Fr. /42

As noted by Turian [147] the coloration of this species is not due to carotenoids: the pigment extracted from it is strongly hypophasic and, in the visible range, shows absorption band very rounded around 440 nm (in ethanol).

2.4 Calocerales

Calocera viscosa Fr. ex. Pers.

Turian [147] characterized in this fungus: β-carotene, major

pigment, and a hydroxy β-carotene which, because of its solubility, he assimilated to cryptoxanthin. Between these two, a very minor red band became individualized, but was too small in content to be studied; on the basis of its color and chromatographic behavior this author inclined to feel it was lycopene.

Except with respect to this last pigment, our results check his data. The spectrum for the total extract is that of β-carotene; from this identification we calculated a total carotenoid content of 0.0037% of dry weight. Ergosterol is also present (O.D. 282 / O.D. 450 = 4).

Column chromatography permits separation of:

- a major fraction, eluted by petroleum ether; its spectrum is that of β-carotene, representing 75% of total carotenoids. Rechromatography shows, however, in the form of traces, very minor yellow bands with the spectrum of β-carotene, but proving to be more polar than γ-carotene with paper chromatography (hydroxy β-carotene esters?) and a red fraction, too small to be studied;

- two yellow fractions, elucted respectively by 5 and 10% ethyl ether in petroleum ether, too small to be studied;

- a yellow fraction, eluted by 15% acetone in petroleum ether and possessing the spectrum of β-carotene; it proves to be heterogenous with paper chromatography (S&S 287) but its major component has an R_f of 0.25 (solvent: petroleum ether) and 0.67 (1% acetone) corresponding fairly well to the values in the literature for cryptoxantin (respectively 0.29 and 0.62); content: approximately 5% carotenoids. /43

0.0037%	β-carotene	75%
	cryptoxanthin(?)	
	approximately	5%
	several pigments:	traces

Dacryopinax spatularia (Schw.) Martin

The carpophores analyzed were gathered in the Central African Republic by Professor Boidin. Acetone extracts the pigmentation, which is shown to be epiphasic and the solution thus obtained has a spectrum very close to that of β-carotene. Going by the

absorption of this pigment, the carotenoid content of this fungus is 0.023% of dry weight.

Column chromatography permits separation of:

- β-carotene, representing 49% of total carotenoids;
- γ-carotene (16%);
- a fraction with the spectrum of β-carotene, eluted by the ethyl ether mixture; petroleum ether 3:1. Here there is a great deal of contamination by colorless impurities so the polarity cannot be accurately determined by analytical chromatography, but seems to be close to lycopene. After saponification, two bands separate in paper chromatography, of the same color, and whose R_f (0.6 and 0.08 with 2% acetone in petroleum ether, 0.7 and 0.24 with 5%) support the hypothesis of one mono- and one dihydroxy β-carotene (cryptoxanthin 0.62, zeaxanthin 0.09 and 0.3 respectively). On the chromatogram, these two pigments appear in the ratio of 2 (least polar) to 1, i.e. approximately -24 and 11% of total carotenoids.

0.023%	β-carotene		: 49%	
	γ-carotene		: 15%	
	hydroxy-β carotenes	approx.	: 24% (mono-)	as esters
		approx.	: 11% (di-)	

Femsjonia luteo-alba Fr. /44

The yellow color of the ether-petroleum solution obtained corresponds only to the edge, in the visible range, of a noteworthy absorption in the UV.

3. GASTEROMYCETES

3.1 Lycoperdaceae

Scleroderma aurantium Vail

The yellow pigmentation of this fungus proves to be strictly hypophasic; its color corresponds to the edge of very intense absorption in UV with a slight inflection at 392 nm (in acetone).

3.2 Phallaceae

Anthurus aseroiformis Mc Alp

The majo- carotenoid in the star-like fructification of this fungus, crystallized in the cytoplasm of its cells, was identified by Eagle [41] and Turian [146] as lycopene. The spectrum of the untreated extract is, in fact, very near to that of lycopene; on the basis of its specific absorption the total carotenoid content proves to be 0.42% of the dry weight. Chromatography permits separation of the neurosporene and the lycopene (ratio: 1:4), identification of which was checked by cochromatography (on S&S 287 paper) with the reference compounds.

0.42%	Lycopene	approx. 80%
	Neurosporene	approx. 20%

4. HYMENOMYCETES

4.1 Hydneae

Hydnum repandum Lin. ex Fr. and H. repandum var. rufescens Pers.

In both cases, the yellow coloration extracted by acetone proves to be strictly hypophasic, and petroleum ether shows only, in UV, the absorption spectrum of ergosterol.

4.2 Corticieae /45

Phlebia radiata Fr.

The orangy coloration of this species is not due to carotenoids: the slight inflections shown, in the visible range where these pigments absorb, on the ether-petroleum spectrum correspond to slight chlorophyll contamination in the specimen.

Hymenochaete mougeotii (Fr.) Massee

The brown coloring (in a concentrated solution) released by this fungus in acetone proves to be strictly hypophasic. The overall spectrum - not taking into account chlorophyll contaminating pigments - shows an inflection at 392 nm in acetone and 400 nm in pyridine. The ergosterol peaks cannot be detected in the epihase spectrum.

Aleurodiscus amorphus (Pers.) Rabenh ex Schroet

Despite the brilliant orangy coloring of this fungus, the visible spectrum of the ether-petroleum spectrum it provides can be attributed to the inevitable chlorophyll contamination of the species. In UV, on the other hand, the ergosterol peaks can be seen.

Peniophora aurantiaca (Bres.) Hoehn. and Litsh.

The cytological location of the pigments of this fungus (orange droplets disseminated through the cytoplasm) would lead one to suspect that they are carotenoids in fairly large quantities; the relative abundance of this species made a study of its pigments particularly favorable. The samples came from Samoëns (Haute-Savoie) where they were gathered in September 1965 and 1966, each batch being analyzed separately.

Acetone extracts all the pigments from the fungus, and they prove to be entirely epiphasic. The total spectrum for the extract peaks at 452 nm with a clear inflection at 474 nm; the ergosterol peaks may be recognized in the UV. On the basis $E^{1\%}_{1cm}$ at 452 = 2300, the quantity of carotenoids is estimated at 0.037% of dry weight.

Since experimentation has shown [8] both considerable lipid contamination and the presence of pigments which are very strongly absorbed by alumina, several processes of analysis have been used: /46

1) Saponification of the total extract, or various fractions of it after isolation.

2) Fractionation of the total extract, either directly by chromatography with alumina (more or less deactivated by hydration: 3% of water - activity II, as in the general case - or 12% water - activity IV to V) or by separation of the pigments according to their solubility (method: the methanol saponification solution is brought together just with petroleum ehter then with ehthyl ether; or division between petroleum ether and 90% methanol; or counterflow between petroleum ether and 95% methanol). The various fractions are then subjected to chromatography on an adsorbant related to their polarity [8].

We also made qualitative analyses of the whole extract by thin-layer chromatography, before and after saponification, in the presence of control substances. The results of these various experiments were in full agreement.

Six pigments were isolated:

- β-carotene (eluted over alumina II by 5% ethyl ether) representing 26% of the total pigments;

- γ-carotene (eluant: 10% ethyl ether) : 2%. The identification of these two pigments was checked by cochromatography with the reference compounds on a thin layer of silica gel [8] and on S&S 287 paper;

- a yellow pigment, γ_{Max} 404, 426, 450 nm in petroleum ether, eluted by 15% ethyl ether; in the absence of reference compounds and because of the very low content (1% of total carotenoids on the arbitrary basis of $E^{1\%}_{1cm}$ = 2500 at 426 nm) it was not possible for us to go further with characterizing this pigment. In fact, since there is only slight chlorophyll contamination of the specimen, it is not even certain that it is a fungus;

- echinenone (eluted by 20% ethyl ether) representing 18% of the carotenoids present; its identification was checked by cochromatographies with the product of synthesis on S&S 287 paper (solvent: 1% acetone in petroleum ether) and on a thin layer of silica gel (solvent: benzene 85, ether 10, methanol 5); /47

- a pigment whose spectrum is very close to that of cantha-xanthin, but which proves to be slightly less hydrophilous (partition coefficient: 56:44 compared to 50:50) and less polar, as with cochromatography it separates clearly from the control cnathaxantin; content (on the basis of the specific absorption of the latter pigment): 15% of total;

- astaxantin, identified after saponification by cochromatography with contrl astacin; content: 35% of carotenoids.

0.037%	β-carotene	26%
	γ-carotene	2%
	"P 426"	1%
	Echinenone	18%
	Di (?) keto-β-carotene	15%
	Astaxantin	35%

Peniophora quercina (Pers. ex Fr.) Cooke

This fungus proves to be carotenogenic; the total spectrum has, next to an intense absorption in the UV (where we see the

ergosterol peaks as inflections) by the side of shoulders in the carotenoid absorption region (452 and 470 nm), peaks showing fairly substantial chlorophyll contanimation; this is confirmed by absorption at 670 nm but the ratio of absorptions in the blue and red shows that the greater part of the carotenoids present is of fungal origin. On the basis $E^{1\%}_{1cm}$ at 452 nm = 2300, the total carotenoid content amounts to 0.003% of the weight of the fungus (as deduced from the contamination).

Two analyses were performed by alumina column chromatography, the first alumina with activity II, the second more strongly deactivated (12% water) to facilitate elution of the most polar xanthophylls. The qualitative results were identical, so we will show the quantitative results of the second.

Separated and identified:

- β-carotene (identified by cochromatography on S&S 287 paper), representing 34.3% of the total carotenoids present;

- echinenone (identified by cochromatographies before and after saponification, on S&S 287 paper and of a thin layer of silica gel): 3.2%

- astaxantin (inseparable, after saponification, from the control astacin): 62.5%.

In view of these results, we might ask whether the violet color of this fungus is due to carotenoids being superimposed over other kinds of pigments, or to the presence of astaxantin in a combined form, as with the shells of certain crustaceans.

	β-carotene	34.3%
0.003%	echinenone	3.2%
	astaxantin	62.5%

Peniophora hydnoidea (Pers. ex Fr.) Donk /48

The spectrum of the natural extract of this fungus, both in the visible and in the UV, is very similar to that given by P. quercina. Two fractions were separated: β-carotene and, at least twice as abundant, a red pigment very strongly retained by alumina, hypophasic and, in the visible range, a rounded spectrum pattern with only one absorption peak around 470 nm in hexane. According to these properties, and the analysis results on other

species of Peniophora, we can identify these second pigment with astaxanthin.

β-carotene: approx. 30%
astaxantin: approx. 70%

4.3 Clavaria

/49

Pistillaria micans Fr.*

Five small Petri dishes (diam. 10cm) containing maltagar medium are seeded with this species and placed at 25°C in a glass oven. After two weeks, pinkish-red pustules appeared all around the shoot and this color developed into a fawn shade which attenuated gradually until it disappeared. The mycelium and conidia were removed at the time when pigmentation was pinkish-red. With the aid of a spatula, the fungus was detached and transferred to acetone. This operation inevitably requires agar and the dry weight of the specimen being analyzed cannot thus be determined.

Acetone takes away all coloration, which passes entirely into petroleum ether. This epiphase is evaporated until dry and dissolved in hexane under UV; this solution, in the visible range, shows a clear inflection at 473 nm, a middle peak at 499 nm, and a secondary peak at 533 nm. Both spectrum and solubility indicate that these are carotenoids. In the UV we recognize the ergosterol spectrum absorption, ten times more intense than that of the pigments.

Chromatography:

- Two yellow-orangy bands, tenuous and diffuse, are eluted by 5 to 15% ethyl ether in petroleum ether. The very low content of these two pigments did not enable us to study them; chromatography behavior and color led to the belief that these were β- and γ-carotenes.

A violet band, turning to red during migration, is eluted by 30% ethyl ether. The spectrum of this fraction has three peaks, at 470, 496, and 529 nm. The position of the absorption maximum and the pattern of the spectrum correspond to no pigment known to

*We are grateful to M. J. Berthier, Maitre-Assistant, who procured cultures of this species for us.

us during this analysis; the results later obtained by N. Arpin and S. Liaaen Jensen [11] on Cookeina sulcipes have since led us to believe that this is an ester of torularhodin.

- At the top of the column remains, strongly absorbed, a substantial red band which cannot be eluted by either ethyl ether, acetone, or ethanol; however, addition of 2% acetic acid to the latter solvent (alkaline methanol) caused a partial shift of coloration. The spectrum in hexane is similar in pattern to that of the preceding fraction, namely to that of a monocyclic carotenoid; the peaks are located at 466, 492, and 526 nm. The results we later obtained upon analysis of red yeasts led us to believe that this is torularhodin, partially isomerized during the process of analysis. /50

β- and γ-carotenes (?):	traces
ester of torularhodin	
torularhodin	major

Clavaria helicoides Pat. and Dem. var. robusta Dorner*

The acetone extracts most of the color from this fungus, which proves to be almost wholly epiphasic. The solution in ether-petroleum has, in the visible range, peaks at 425, 449, and 476 nm and in the UV the spectrum of ergosterol (O.D. 282 / O.D. 449 = 1.5). On the arbitrary basis of $E^{1\%}_{1cm}$ = 2500 at the absorption maximum, the total carotenoid content is 0.033% of the dry weight.

Chromatography on an alumina II column permits separation of:

- a yellow-orangy fraction, very large; its spectrum and chromatographic behavior (elution with 5% ethyl ether in petroleum ether) led to its identification as β-carotene, which is verified by cochromatography (on S&S 287 paper) with the reference compound; the β-carotene accounts for 80% of the pigments present in the ether-petroleum solution.

- a small red fraction, eluted by 50% ethyl ether. Its spectrum (γ_M 470, 494, 522 nm with a monocyclic carotenoid

*We would like to thank M. J. Berthier who determined this species, new to Africa.

pattern) and its polarity (R_f = 0.32 on S&S 287 paper, solvent 2% acetone in petroleum ether) argue in favor of it being dehydro-3, 4 torulene (489.5, 519.5 nm, R_f = 0.35). This pigment (on the basis $E^{1\%}_{1cm}$ = 3000 at 492 nm) accounts for 1.5% of the total carotenoids. /51

- a pink fraction, eluted by 5% acetone in petroleum ether. The spectrum reveals its heterogeneity, but the predominant peaks are those of another monocyclic carotenoid, dehydro-2' plactaniaxantin [10]; cochromatography with this compound confirms this identification. Content: about 2% of carotenoids present.

- finally, two yellow bands are eluted by 10% and by 20% acetone; their spectrum and polarity indicate that they are likely to be hydroxy-β-carotenes. Given their slight content, and the chlorophyll contamination of the specimen, it is not certain that they are of fungal origin; we did not pursue study of these fractions.

0.033%	β-carotene	80%
	dehydro-3, 4-torulene (?)	1.5%
	dehydro-2' plactaniaxanthin	2%
	hydroxy-β-carotene (?) approx.	10%

Neurophyllum sp.

This species, gathered like the above in the Central African Republic by Professor Boidin, furnished an epiphase which had the absorption peaks of ergosterol only.

Clavaria without carotenoids

For clarity, we are grouping together here the species of Clavaria (in the older and broadest sense of the term) in which a search for carotenoids proved negative.

These are:

Cl. pallida B. & C.
Cl. corniculata Schoef. ex Fr.
Cl. aurea Schoef. ex Fr.
Cl. formosa Pers. ex Fr.
Cl. fistulosa Fr.
Cl. truncata Q.
Cl. pistillaris Lin. ex Fr.

In all cases the yellowish coloration extracted by ethanol /52
corresponds only to one side of absorption in ultra-violet, with a slight inflection at 340 nm for Cl. pallida and Cl. corniculata, 390 nm for Cl. aurea and Cl. formosa. This colorant, extracted by aceton, is shown to be strickly hypophasic, and petroleum ether has only, in all cases, ergosterol absorption peaks.

4.4 Cantharellaceae

Cantharellus konnadii (R. Maire) Kühn.-Romagn.

The extract by acetone of this fungus is saponified; the epiphase obtained has only, on one side of a very intense absorption in the far ultra-violet, peaks at 282, 292, 303, and 319 nm which remind one of the colorless compounds extracted from C. cornucopioides and C. tubaeformis.

Cantharellus cornucopioides (Fr. ex Lin.) Kühn.-Romagn.

Turian [147] found no carotenoid in C. cinereus, and we were therefore hardly surprised by the epiphase coloration, which in the visible range of the spectrum has a mixture of neuroporene and lycopene; however, the carotenoid content of this species is only 0.0006% of its dry weight. The whole spectrum also has peaks at 280, 300, and 315 in the ultra violet.

The abundance of lipid contaminants rendered saponification necessary, so that the two phases obtained on demixing at the end of this reaction were studied separately.

The hypophase, orange-russet in color, has absorption peaks in the ultra violet alone, at 277, 287, 300, and 315 nm. The coloration observed pertains only to the side of the peak, in the visible range of this absorption. After acidification with acetic acid, the color turns greenish-yellow and is principally epiphasic; the absorption peaks in UV (in a hexane solution) are the same as above, and the color change observed is shown only by a change in slope of the spectrum when approaching the visible.

The epiphase presents, in UV, ergosterol peaks and, in the visible range, peaks at 416, 440, 470 and 500 nm. Analysis is /53
conducted qualitatively by paper cochromatography in the presence of control compounds, showing that it is composed of neurosporene and lycopene; semi-quantitatively, spectroscopic determination of absorption of the two pigment after their chromatographic elution and calculation based on the ratio of the peak heights in the full

spectrum both indicate that neuroporene and lycopene are present in a ratio of 3:2.

0.0006%	Neurosporene:	approx. 60%
	Lycopene :	approx. 40%

Cantharellus tubaeformis Fr.

The first analysis of the carotenoid contents of this species was done in 1937 by Willstaedt [162]. This author isolated lycopene and a major pigment which he believed to be 8'apo-β-carotenal. In 1960, Turian [147] showed that the latter pigment was in fact neurosporene, and also showed the presence of a "minor oxycarotenoid."

The full spectrum is very close to that of neurosporene; according to its specific absorption, the total carotenoid content amounts to 0.02% of dry weight [51].

After 12 hours saponification, required by the presence of large quantities of lipids, the unsaponifiable fraction is extracted by petroleum ether and concentrated under low pressure: there then appears a large white precipitate of ergosterol, eliminated by filtration.

With alumina I column chromatography, seven fractions are eluted in turn; their properties are shown in the tables XIII and XIV with their deduced identification. Fraction 2 has two secondary peaks at 318 and 329 nm (E_{329}/E_{432} = 0.45) in petroleum ether: this is the double cis peak of aliphatic carotenoids [166]; identification of fractions 4 and 5 was checked by cochromatography with the reference compounds; fraction 6, minor and contaminated with very large quantities of ergosterol, could not be studied further: its spectrum seems to show presence of an aliphatic chromophore (about 8 conjugated double bonds) and adsorption argues in favor of a mono-hydroxylated derivative.

The fraction 7 spectrum is exactly identical to that of ζ-carotene. The chromatographic properties and the partition coefficients (32:68, 54:46, 86:14 between hexane and methanol at 95, 90, and 85%) are in favor of a dihydroxycarotenoid with a short conjugated polyenic chain; acetylation is negative, silylation positive, indicating the tertiary nature of these hydroxyls. We thus proposed for this pigment the structure dihydroxy-1,1' tetrahydro-1, 2, 1', 2' ζ-carotene, since it is much more likely

TABLE XIII

Chromotographic properties of Cantharellus tubaeformis caratenoids [51].

Fraction	Eluant on Al_2O_3 II	Gel G 0%*	Gel G 0%**	Gel G 2%**	RF values on: 0%*	2%*	5%*	S & S n° 287 paper 10%*	15%*
1	Ether de pétrole	0,1	0,32						
2	Ether de pétrole		0,25	0,84					
3	5 % +		0,24	0,84					
4	15 % +		0,18	0,80	0,89				
5	3 % x			0,72	0,48				
6	15 % x			0,10					
7	5 % méthanol in benzène			0.0		0,08	0,30 (1)	0,60 (2)	0,89

\+ Ethyl ether in petroleum ether (boiling pt. 40-60°C)

x Acetone in petroleum ether (boiling pt. 40-60°C)

** Acetone in petroleum ether (boiling pt. 100-200°C)

(1) 0.98 after silylation

(2) Zeaxanthin : 0.56

TABLE XIV

Spectrum properties and distribution of Cantharellus tubaeformis carotenoids [51].

Fraction	λ_{max} (P.E.) nm.	Identification	% total caroténoïdes
1	331 348 361	Phytofluène	1,4
2	409 432 461	cis-Neurosporene	2
3	378 400 425	ζ-carotène	1,6
4	415 439 468	Neurosporène	72.0
5	445 472 503	Lycopène	8,8
6	398 420 446	?	1
7	378 400 425	DiOH- ζ-carotène	13,2

that there is a substitution at 1 than at 5. This is a new natural compound [51].

We should finally report that, with the first fraction, a colorless compound was eluted whose spectrum shows three very sharp peaks at 287, 300, 315 nm in petroleum ether, similar to that of a non-carotenoid (steroid?) compound extracted in the manner of C. cornucopioides above.

Cantharellus lutescens (Pers.) Kühn.-Romagn.

Analysis of a small sample of this species enabled us to identify neurosporene, representing approximately 45% of total carotenoids, lycopene - 50% - conforming to previous results [162, 147] and two minor fractions probably the same as the last two pigments of C. tubaeformis.

Neurosporene	:	approx. 45%
Lycopene	:	approx. 50%
Two pigments	:	approx. 5%

Cantharellus friesii Q.

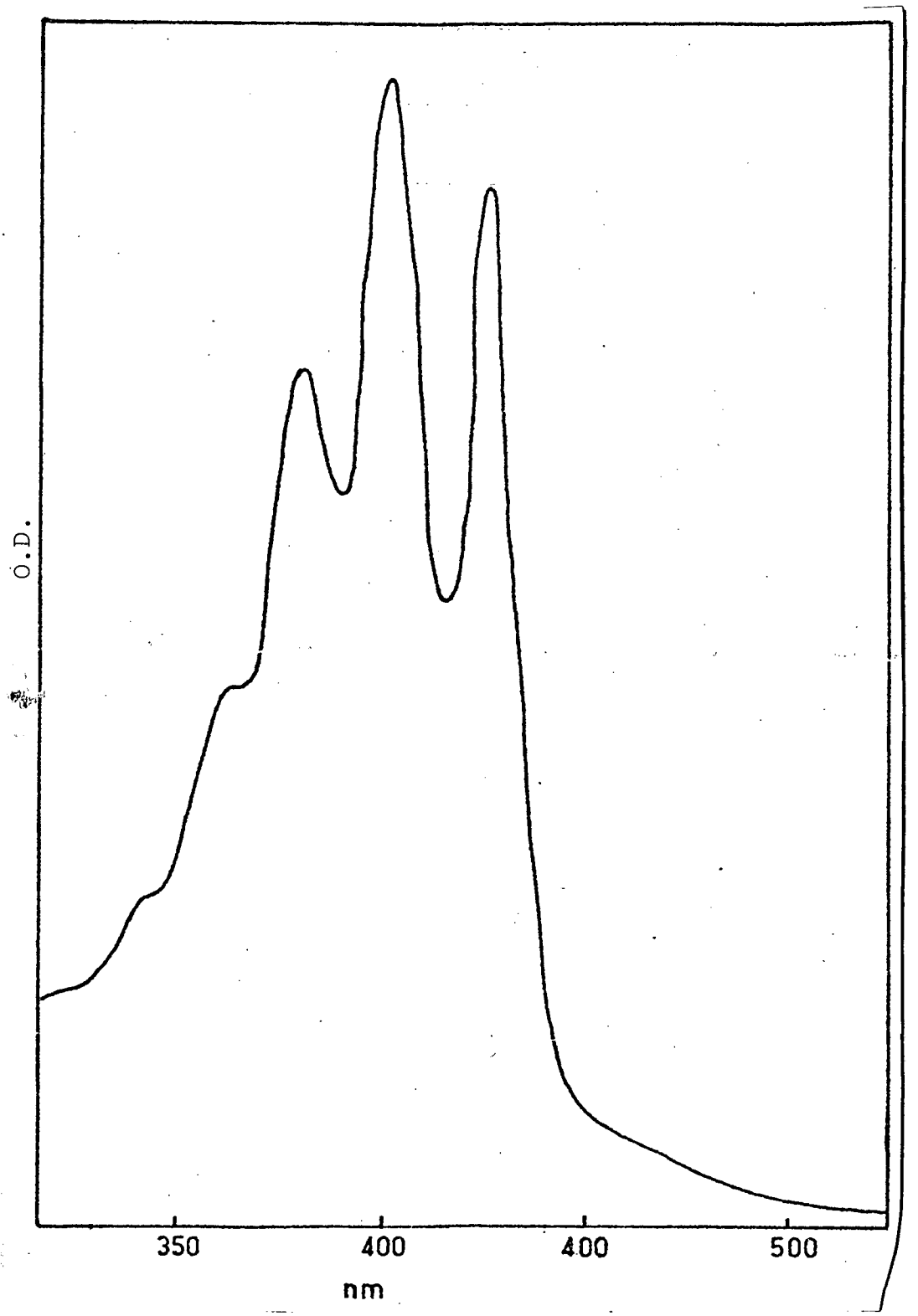
The sample is treated with acetone until a colorless extract is obtained; the extraction residue remains slightly fawn in color. The solution in ether-petroleum is saponified overnight, and the two phases which separate at the end of this reaction are studied separately.

The hydro-methanolic alkaline phase is colored orangy-russet by strictly hypophasic compounds under these conditions; they are epiphasic (and yellow) after acidification. The spectrum (in ethyl ether, after several rinsings in water to eliminate the acetic acid) presents, on the side of an absorption much more intense in the furthest UV, a shoulder at 281 nm and a peak at 317 nm.

The epiphase has ergosterol peaks in ultra violet and peaks at (425), 448, and 475 nm in the visible range. The following are separated by chromatography: /55

- β-carotene;

- a pigment with spectrum close to that of echinone, but with a partition coefficient of 86:14 (instead of 93:7);


O.D.
350
400
400
500
nm

- a salmon pigment with the spectrum of canthaxantin but a coefficient partition of 55:45 (instead of 50:50), thus reminding one of a minor pigment of Peniophora aurantiaca.

Cantharellus cibarius Fr., variety: amethystinus Q

Although the type for this species showed Willstaedt [162] fairly rich carotenogenesis in which β-carotene predominated, this variety gave us a colorless epiphase, showing only ergosterol peaks in the shape of shoulders. This variety thus differs from the type not only by the development of pigments other than carotenoids, but by the disappearance of the latter.

4.5 Agaricales

Omphalia chrysophyla Fr.

It was particularly interesting to study the pigmentation of this Agaricale which has many characters in common with the Chanterelles [97]. Alumina I column chromatography separates [50]:

- β-carotene (representing 87% of total carotenoids)
- γ-carotene (11%)
- lycopene (0.5%)
- torulene (1.5%)

identification of which was verified by cochromatography (on S&S 287 paper) with the reference compounds before and after saponification.

Omphalia abiegna Bk. Br.

The epiphase obtained from this species is colorless; on the side of a very large absorption in UV, only inflections corresponding to ergosterol peaks can be recognized.

Phyllotopsis nidulans (Fr. ex Pers.) Gilb.-Donk. /56

This Agaricale is carotenogenous: the epiphase presents, in the visible range, peaks at (428), 454, and 500 nm; on the basis of an average $E^{1\%}_{1cm}$ equal to 2500 at 454 nm, the carotenoids represent 0.028% of the dry weight of this fungus. Ergosterol is

also present (O.D. 282 / O.D. 454 LL).

Alumina II chromatography separates:

- β-carotene (representing 58% of total carotenoids)
- γ-carotene (29%)
- neurosporene (0.5%)

and identification of these fractions was checked by cochromatographies (on S&S 287 paper) with reference pigments.

- echinenone (8%), identified according to its spectrum which, after treatment of the pigment with lithium-aluminum hydride, gives that of β-carotene; this fraction proves to be contaminated by small quantities of a pigment of the same color as canthaxanthin, but clearly less polar in paper cochromatography, recalling a minor compound in Peniophora aurantiaca and perhaps Cantharellus friesii,

- an impure fraction, eluted by 20% acetone in petroleum ether and having substantially the same spectrum as β-carotene: this is likely to be a hydroxylated derivative of β-carotene; content: about 1%.

- astaxanthin (3.5% of the total after extrusion of the column, doubtless more in the fungus, due to particularly large losses during isolation), identified by its chromatographic behavior and spectrum, which by reduction of the pigment gives that of β-carotene, and cochromatography (on G silica gel) with reference astacin after saponification.

β-carotene	58%
γ-carotene	29%
Neurosporene	0.5%
Echinenone	8%
Hydroxy-β-carotene (?)	1%
Astaxanthin	3.5%

Clitocybe venustissima (Fr.) Sacc. /56

The first analysis of this fungus [5] showed that its pigmentation consists wholly of carotenoids, accounting for 0.17% of its dry weight. The following were separated:

TABLE XV

	Dihydroxy- -carotène (VIII)	Neurosporène (IV)	Lycopène (V)	γ -carotène (XVI)	Torulène (XVII)	3,4-déhydro torulène	Torularhodin (XXV)	2'-dehydro plectaniaxanthin (XXII)	β -carotène (XXVII)	Cryptoxanthin (XXVIII)	Zeaxanthin (XXIX)	Echinenone (XXXII)	Astaxanthin (XLIII)	Kéto-(β)-caroténoïdes not identified	Xanthophylls not identified	Caroténoïds not identified
Rhodotorula aurantiaca (Saïto) Lodder				+	+		(+)		+							
Rh. glutinis (Fres.) Harrison				+	+		+		+							
Rh. sannlei (Cif. et Red.) Lodder				+	(+)		+		+							
Sporidiobolus johnsonii Nyland				+	+		(+)		+							
Ustilago zeae (D.C.) Cda.			+													
Ust. scabiosae (Sow.) Wint.			+	+												
Calocera viscosa Fr. ex Pers.									(+)							
Dacryopinax spathularia (Schw.)Martin									(+)							
Anthurus aseroiformis Mc Alp.		+							+							
Peniophora aurantiaca (Bres.) Hoehn.et Litsch.				+					+							
P. quercina (Pers. ex Fr.) Cooke									(+)							
P. hydnoides (Pers. ex Fr.) Donk									(+)							
Pistillaria micans Fr.				?			(+)		+			+				
Clavaria helicoides Pat.et Dem.var.robusta Corner						?			(+)							
Cantharellus cornucopioides (Fr.ex L.)K'hn.-Romagn.			+	+												
C. tubaeformis Fr.			(+)													
C. lutescens (Pers.) Kühn.-Romagn.			+	(+)												
C. friesii Q.																
Omphalia chrysophylla Fr.				+	+				+							
Clitocybe venustissima (Fr.) Sacc.				+	(+)				(+)							
Phyllotopsis nidulans (Fr. ex. Pers.) Gilb.-Donk			+	+					(+)			+				

- β-carotene (accounting for 8% of total carotenoids)

- γ-carotene (approximately 75%)

Identification of these two pigments, based on their spectrum and chromatographic behavior, was confirmed by cochromatography of a thin layer of G silica gel (solvent: 2% acetone in petroleum ether, boiling point 100-200°C) with the products of synthesis.

Above γ-carotene, two very pale bands, pink and yellow, were observed. They were too small to permit study.

A second analysis, on a still smaller sample, was performed by cochromatographies (on S&S 287 paper) of the total extract, before and after saponification, with the reference compounds. It fully bore out the results of the first analysis and in addition enabled us to identify the small pink band as lycopene.

β-carotene		8%
γ-carotene	approx.	75%
2 pigments incl. lycopene:	approx.	17%

Clitocybe dealbara Fr. ex Sow.

The epiphase spectrum has only the specific peaks of ergosterol.

Hygrophorus puniceus Fr.

The visible range spectrum of the acetone extract of this fungus shows a very broad and rounded absorption peak around 416 nm, with slight shoulders at 455 and 480 nm. This coloration proves to be strictly hypophasic, the epiphase showing only the absorption peaks of ergosterol.

Poromycene sp.

This species (from the Central African Republic), a brilliant orange, when treated with acetone gives only a pale epiphasic color. The total spectrum, in petroleum ether, has on the side of a very intense absorption in the UV, small peaks at 322, 338, 382, 405, and (434) nm. /58

Preferential solubility experiments were then attempted, under partition coefficient measurement conditions. We show below

the spectrum of the various fractions obtained, and from what wavelength these measurements were done. All the peaks are to the side of a very intense UV absorption:

Petroleum Ether	/	Methanol, 95%
(320 : 323, 338, 385, <u>408</u>, 433 ; (O.D. : 0,2)		(270 : 295, 309, <u>329</u>, (338), (408). (O.D. : 1,2)
P.E.	/	MeOH 95% + 10% KOH
(300 : 326, 345, <u>364</u>, 383, (408)(433); (O.D. : 1)		(270 : 292, <u>339</u>, 355. (O.D. : 0,7)
P.E.	/	MeOH - KOH as above + acidification
(300 : 326,(345), <u>363</u>, 383, (408)(433); (O.D. : 0,35)		(300 : 323, 340, 363 (O.D. : 0,35)
P.E.	/	MeOH acidified by HC.l
(320 : 322, <u>362</u>, 384, 405, (432); (O.D. : 0,4)		(260 : 363 (O.D. : 0,2)

Such variations in the spectral absorption and solubility as a function of pH imply that these pigments are not carotenoid in nature. However, alumina II column chromatography was attempted. Petroleum ether, progressively enriched with 10 to 20% ethyl ether, separates four yellow bands each with the same absorption peaks: 292, 307, 323, 342, 363, 381, (404), and 430 nm, the relative height of these peaks and the fineness of the spectral pattern always varying from one fraction to another: the 363 nm peak is major for the first two fractions, then the 323 nm becomes the most prominent, this absorption zone remaining well individualized as compared to that of the further UV. This would tend to show that, although different bands individualized on the column were satisfactorily separated, we have to do with a mixture whose properties vary in the course of elution.

These results, however, confirm that these yellow fat-soluble pigments are not carotenoids.

Lactarius tornimosus Fr. ex Schaef /59

β-carotene having been identified in the mycelium of this species [91], it was interesting to analyze its fructification. The colorant extracted by acetone proves to be strictly hypophasic, and the epiphase has only ergosterol absorption peaks.

Violet Cortinaria

The pigments responsible for this coloration are cytoplasmic, like the carotenoids; their color, however, would seem to make them not carotenoids. Against this are two facts: 1) pigments of this family and purple in color were recently isolated from fungi [10], and 2) the bonding of certain carotenoids with, for example, proteins can alter their color considerably, as in the case of the pigments of certain crustaceans: it was thus interesting to analyze representatives of this group. The following species were studied:

Cortinarius evernius Fr.
C. " " traganus Fr. ex Wienn.
C. " " albo-violaceus Fr. ex Pers.
C. " " caerulescens Schaef.
C. " " praestans Cord.

The acetone in which the specimens were kept is always very turbid. It is greenish-yellow for C. traganus and C. caerulescens, but virtually colorless in the other cases. This coloration, hypophasic, corresponds only to the side of the UV absorption, with no well-defined peak in the visible range. On partition, only a very substantial emulsion appears, and the epiphase has only, in each case, the ergosterol spectrum. In the case of C. caerulescens, the hypophase was evaporated until dry; the residue, a yellow-red color, dissolves neither in ethanol nor in ethyl ether nor in N,N'-dimethyl formamide. It dissolves partially in water (giving a cloudy appearance) and totally in 50% ethanol. None of these species color with acetone, ethanol, methanol, benzene, or N,N'-dimethyl formamide. Only alkaline methanol (95% methanol containing 10% potassium) gives something of a yellow coloring (side of UV band), strictly hypophasic. When acidified (with acetic acid) the shade turns pale greenish-yellow (resulting in a change in the slope of the spectrum), without its hypophasic character being altered. /60

TO SUMMARIZE: the following species did not show carotenoid pigments: /60

Auricularia auricula-Judae L. ex Fr.
Guepinia helvelloides D.C. ex Fr.
Femsjonia luteo-alba Fr.
Scleroderma aurantium Vail.
Phlebia radiata Fr.
Hymenochaete mougeotii (Fr.) Massee
Aleurodiscus amorphus (Pers.) Rabenh. ex Schroet.
Hydnum repandum L. ex Fr. and its variety
rufescens Pers.
Clavaria pallida B. et C.
Cl. corniculata Schaef. ex Fr.
Cl. aurea Schaef. ex Fr.
Cl. formosa Pers. ex Fr.
Cl. truncata Q
Cl. pistillaris L. ex Fr.
Neurophyllum sp.
Cantharellus konradii (R. Maire) Kühn.-Romagn.
Canth. cibarius Fr. var. amethysteus Q.
Omphalia abiegna Bk.-Br.
Clitocybe dealbata Fr. ex Sow.
Hygrophorus puniceus Fr.
Poromycena sp.
Cortinarius evernius Fr.
Cort. caerulescens Schaef.
Cort. albo-violaceus Fr. ex Pers.
Cort. traganus Fr. ex Weinm.
Cort. praestans Cordier
Lactarius torminosus Fr. ex Schaef.

Table XV indicates the pigment contents of the carotenogenous species studied.

CHAPTER IV

DISCUSSION AND CONCLUSION /61

Due to our very target in this work, our results taken as a whole are too widely scattered among the multifarious taxons of Basidiomycetes to offer a topic for general discussion. With the exception of a particularly frequent trend of carotenoid loss, the data gathered here do no more than confirm the table of fungal carotenogenesis and its singularities that we drafted in the Introduction to this thesis. Here, we will confine ourselves to looking at the contribution of this study to a clarification of taxonomic and phyletic relationships in two groups of fungi:

- The carotenogenic yeasts and their relationships with Heterobasidiae, particularly Ustilaginales.

- The higher Hymenomycetes, in particular the problem of the position of the Chanterelles as a possible link between the Agaricales and certain Aphyllophorales.

1. RELATIONSHIPS BETWEEN CAROTENOGENIC YEASTS AND HETEROBASIDIAE

We referred above to the elements showing the close connection between asporogenous carotenogous yeasts (Rhodotorula) and ballistosporogenous carotenogenous yeasts (Sporobolomyces). The chain of reasoning on the system position of Sporobolomycetaceae has been summarized by Lodder, Sloof, and Kreger-van Rij [113]. The fungi in this family (Sporidiobolus and Bullera, as well as Tilletiopsis and Itersonilia, the latter two, since they actually form mycelia not considered as yeasts) are characterized by the appearance, on apparently vegetative cells, of a sterigma at the top of which a spore is differentiated and then ejected by a mechanism similar to that of basiodiospore abstriction in the Hymenomycetes. It is thus tempting to make such a comparison, /62 especially with the Heterobasidiae, in which the basidium divides into elements each giving rise to a spore; the validity of this viewpoint is essentially determined by identification of the ballistospore to the basidiospore, fundamentally linked to sexual reproduction. This is why it was not at first considered that the Sprorbolomycetaceae belonged to the Basidiomycetes, since the spores of the former were considered to be conidia. In fact, the vegetative cells which give rise to them are uninucleate

(contrarily to the secondary mycelium of Basidiomycetes) and no caryogamy was observed. However, Sainclivier (1951), pointing out that caryogamy was fairly frequent in the yeast-like cells of Sporobolomyces, concluded that it regularly preceded spore formation. Thus, he supported the notion that Sporobolomycetaceae are Basiodiomycetes. Thus, the monospecific Sporodiobolus, very close to Sporobolomyces, with which it differs only by producing a true looped mycelium, was described as being a Heterobasidium; and in his Traité de Botanique Systématique, Chadefaud [27] classifies the Sporobolomycetaceae with the Basidiomycetes.

Although our results confirm the close affinity of Sporobolomyces and Sporidiobolus, their pigmentation being identical, they have no hard argument to add as concerns their position with respect to the Heterobasidia. Indeed, although the two Ustilago studied are carotengenous, their β-carotene-based pigmentation appears "undifferentiated" and, at least under our culture conditions, shows none of the carotenoids (torulene, torularhodin) of the red yeasts, presence of which, crosschecked by other kinds of data, would be a very strong indication of a relationship between these groups. We should remember however that Lederer [101], analyzing the carotenoids of Puccinia coronifera (gathered wild) had found an acid pigment which he found similar to torularhodin, which pigment he himself had characterized in (Rhodo) Torula. Torulene and torularhodin have elsewhere been found in various Discomycetes [26, 107, 11] and the latter, so we believe, in Pistillaria micans, a reduced member of Clavariaceae which develops on dead leaves. (This habitat is not far removed from that of Sporobolomycetaceae.)

2. HYMENOMYCETES

/63

2.1 Generally speaking, the first observation which must be made is the dispersed and limited possession of carotenoid pigments within the various Hymenomycete groups. The following proved to be carotenogeous:

- two clavarioid fungi,
- several cantharelloid species, centered particularly around the genus Cantharellus in its narrowest interpretation,
- a genus of Corticieae (Peniophora),
- three species of Agaricales.

The most general case in the Hymenomycetes is loss of this

family of pigments, retained, and perhaps evolved, only in a few disjointed series.

Furthermore, we may note that, in three genera with no direct connection (Cantharellus, Peniophora, and Phyllotopsis), carotenogenesis leads to accumulation of ketonic derivatives of β-carotene. Rather than viewing this as an ancestral character surviving in patches, we prefer to see it as the result of convergence phenomena. Firstly, the carotenoids accumulated could be of a quite different kind (torularhodin, dehydro-2' plectaniaxanthin), but also representing carbonylated derivatives, here of a monocyclic hydrocarbon skeleton. The appearance of such ketone or carboxyl groups corresponds to fast oxidation of the carotenoid molecule. We have already stated the reasons for thinking that such oxidation is synonymous with evolution: this is continually being confirmed by the study of Discomycete carotenoids, where progress in oxidation of these pigments closely parallels the evolution of macroscopic and microscopic characteristics [9-11]. The bicyclic skeleton of β-carotene, the normal endpoint in biogenesis of carotenes proper, would be the most frequent substrate of this oxidation; particular specialization would then lead to the chains of reaction becoming directed along the path of monocyclic carotenoids. Moreover, comparative distribution of carotenoids within the Ascomycetes and Basidiomycetes leads one to attribute an "undifferentiated" carotenogenesis to the common origin of these two great phyla (major β-carotene, γ-carotene, lycopene, torulene). Now, it is the same pigmentation /64
as that of the species studied here - Omphalia chrysophylla, as we shall see - which appears to be the nearest thing to a link between two groups (in which species accumulating keto-β-carotenoids are found).

2.2 Genus Peniophora sensu stricto

Boidin [18, 19] showed how natural this deliniation is. Carotenoid analysis supports this viewpoint since, despite differences of coloration, the carotenogenesis of P. aurantiaca and P. hydnoidea on the one hand (incarnata group), and P. quercina on the other (cinerea group) is qualitatively very similar (β-carotene and ketonic derivatives and above all astaxanthin). We may, however, note that when we go from P. aurantiaca to P. quercina, the carotenoid content drops by nine-tenths, or nearly, while pigments of other kinds develop.

2.3 Clavarioid Fungi

This taxon is defined only by the very similar macroscopic morphology: the artificial character of this group, revealed by microscopy, is thus hardly surprising, and Corner, for example, recognized twenty-seven genera [31]. The species which concern us are distributed as follows:

Ramaria aurea (Fr.) Quel.
Ramaria formosa (Fr.) Quel.
Nevrophyllum sp.
Ramariopsis Kunzei (Fr.) Donk (= Cl. pallida B.& C.)
Clavulinopsis corniculata (Fr.) Corner
Clavaria helicoïdes Pat. et Demange
Pistillaria micans Fr.
Clavariadelphus fistulosus (Fr.) Corner
Clavariadelphus truncatus (Fr.) Donk
Clavariadelphus pistillaris (Fr.) Donk

Corner, in his revision of all the clavarioid fungi [31], based essentially on the structure and development of the fructifications, defines, above the genus level, only "series" representing the affinities he detects. Ramaria, it seems, with ocherous and ornate spores, would be placed outside of Gomphus /65
(incl. Nevrophyllum); Clavulinopsis and Ramariopsis are neighbors and seem close to Clavaria; Pistillaria would not be far from Clavariadelphus, whose affinities would be looked for rather on the Cantharellus side. Donk [40] includes Ramaria with the Gomphaceae, the other genera cited being assembled in the Clavaricae family, a tribe of Clavarioidea (Clavariadelphus, according to this author, should not be close to the Cantharellaceae, because of the different arrangement of mitoses in their basidia - to which character he attaches more importance than Corner in this case at least, and because of their mode of development and their dark green reaction with ferric sulfate: the latter character in conjunction with others, would bring them closer to the Gomphaceae). The color of the spores when stained by cotton blue, observed by Petersen [125] supports the views of Donk except for this point: the Ramaria spores (at least in the species concerning us here) are effectively similar to those of Gomphus, while they are like those of Clavaria, Clavulinopsis, Clavariadelphus (and Cantharellus), in which the cytoplasm (and not the spore wall) stains with the exception of the fat droplets inside. The greater or lesser coalescence of these droplets distinguishes Clavaria, Cantharellus, and the Clavulinopsis-Clavariadelphus set.

With respect to species that were shown to be bereft of carotenoids, we may note the closeness of the spectrums of the two Ramaria studied (very close according to Corner [31]), and that of the spectrums of Clavulinopsis and Ramariopsis, which appears to be an element favoring the close affinity of these two genera.

The two species here recognized as carotenogenous: Pistillaria micans and Clavaria helicoïdes, have in common their rather exceptional orientation in the direction of monocyclic carotenoids. This is a striking convergence with the Sarcoscyphaceae [9-11]. This appears to us a rather strong argument in favor of their closeness to each other, although, as we have seen, Corner places the two genera in very different series of affinities. We must note the slightly external position of Cl. helicoides in the Clavaria genus (other species of which have red pigments whose nature remains to be specified) by its colored spores and the secondary septation of its hyphae and older basidia; Corner himself, however, brings at least two pink Pistillaria (P. montevidensis Speg. and P. rhodocionodes Corner) together with Cl. helicoïdes. /66

It should be noted that, although Clavulinopsis corniculata showed no carotenoids, the red carotenoid-pigmented Clavaria described by Heim [83] (Clavaria cardinalis Boud. and Pat. = Clavulinopsis miniata (B.) Corner var. sanguinea Corner) has been placed in this genus. Moreover, Corner notes that in this genus the red color seems to be due to carotenoids dissolved in droplets of cytoplasm. The nature of these pigments should be checked by analysis; indeed, in the only species wherein the carotenoid nature of the pigment seems well established (Cl. miniata) the medullar hyphae have the highest coloring; elsewhere the orangy pigments are mainly concentrated in the sub-hymenium. And, especially if their cytological location suggests carotenoids, the same goes for the yellow pigment of Cl. corniculata which, as we have seen, is of a different nature. Moreover these pigments turn clearly green with iodine but are unaltered by concentrated sulfuric acid: as against their position in the cell, their location in the fungus, like certain chemical characters, would seem to make them not carotenoids [31].

2.4 Agaricales

It has long been thought that Agaricales proper (excluding Chanterelles) did not synthesize carotenoids [5]. In fact, although some rare species are carotenogenic, this character is

exceptional. β-carotene has been reported in the mycelium of a Lactaria [91], but the presence of this family's pigments in the fructifications is the case, as far as we know now, for three species only: Phyllotopsis nidulans, Clitocybe venustissima, and Omphalia chrysophylla. Note that Singer classifies the latter two species in the same genus, Gerronema, as the others (Omphalia xanthophylla, for example, whose pigmentation could be of the same kind. But Phylloptopsis and Gerronema seem to have no direct relation, and this dispersion of carotenogenesis, like its extreme /67
rarity, seems to make it a relic surviving only here and there: in general carotenoids have disappeared in Agaricales, giving way to other pigments such as anthraquinone.

2.5 Cantharelloid Fungi

From the beginning, the Chanterelles appear to be intermediate between the Agaricales and the Aphyllophorales: indeed they are agaricoid Hymenomycetes (base and pileus, the latter fertile only on the lower side) but without true gills, the hymenium being, at most, pleated with often dichotomous veins. This morphological definition is clearly too broad to delimit a natural group - indeed species which are irrefutably Agaricales but regressed ones are classified here - and needs further specification. The criterion held to be the essential one is the stichobasidial mode of division inside the hymenium. This character would seem to separate the Chanterelles from the Agaricales where it is unknown; they also differ in other areas (structure of hymenium, method of development) and were therefore often excluded from this order and classified with the Aphyllophoales with which a number of authors sought affinities. Two problems arise here: the relationships within the group so defined (distinction and true degree of affinity between Cantharellus and Craterellus); and the position of the group as a whole, or at least the Cantharellus genus.

Kühner and Romagnesi [99], studying European Chanterelles, distinguish at most two subgenera according to the presence (Cantharellus) or absence (Craterellus) of loops. Romagnesis, of the species studied for carotenogenesis, includes C. cornulcopiodes and C. cinereus. Corner [32], attributing prime importance to the development and structure of the fructifications, and considering the arrangement of the mitotic bundles in the basidia as a secondary character, clearly separates Craterellus (restricted to the most infundibuliform species) together with C. cornucopioides, from Cantharellus (where C. cinereus is close to C. tubaeformis and C. lutescens), as the development of the former appears to him to be relatively close to that of the

Clavaria than that of the true Chanterelles. Donk [40], on the other hand, according greater importance in this case to the disposition of the basidia divisions, considers them as very close and in the same family as the Cantharellaceae. /68

Now, due to both its carotenoid pigments and to other kinds of compounds, C. cornucopioides is, as we have seen, very close to the C. lutescens-C. tubaeformis group, from which, and from our point of view, it differs only by less abundant carotenogenesis with, correlatively, strong development of other kinds of dark pigments. These data argue very strongly in favor of the homogeneity of the Cantharellus genus (ss. Kühner-Romagnesi). In fact, the results obtained would very closely fit C. cinereus, morphologically speaking. This is why - and the problem is an important one - we feel that the results presented here need confirmation. One source of error is to be feared at the determination stage, given the likely heterogeneity (in view of the very low overall content found) of the batch submitted for analysis. The validity of the negative results obtained by Turian [147] for C. cinereus depends, for the same reason, somewhat on the size of the sample analyzed.

It could also be noted that Corner [32] subdivides the Cantharellus genus into several subgenera, including (going by the growth and whether or not there is a cavity in the stipe) Cantharellus (with C. cibarius, C. cinnabarinus, and C. friesii) and Phaeocantharellus (with C. lutescens and C. tubaeformis); but it should be noted that all the species analyzed of the first group accumulate bicyclic carotenoids (β-carotene and ketone derivatives) and those of the second group, exclusively aliphatic compounds. Looking solely at the order of appearance of these compounds during biogenesis one would be tempted to consider the aliphatic carotenoid species as more privitive. These also contain other kinds of (brownish) pigments, and one would also be tempted to postulate from these, a divergence in evolution leading either to C. cinereus or to C. cibarius and thence to C. friesii, with in each case a loss or regression in one of the two types of pigments initially present. However, it seems highly unlikely that the bicyclic carotenoid pathway, developed in certain Chanterelles, is here a rediscovery; also, as already noted in this connection [147] the neurosporene only accumulates, as it would seem, when carotenogenesis undergoes partial inhibition. One might thus think that it is from C. cibarius, whose pigmentation is fairly undifferentiated (and as we shall see is found in a close Chanterelles species) that evolution would have developed, equally divergent, and would have led either to /69

C. friesii or to C. cinereus, but where the C. tubaeformis-C. lutens-cens group marks a regression stage of carotenoids along this second phylum.

Kühner [97], in the accompanying plate illustrating Omphalia chrysophylla Fr. notes the extent to which this irrefutably Agaricale (by growth, gills, and the chiastobasidian mode of divisions in the hymenium) is close to the true Chanterelles by (aside from color and general appearance) its entangled trama, its sporing, and its hymenium poorly defined by the sub-hymenium. However, it was generally taken as read that Canthareaceae should be excluded from the Agaricales, and authors sought rather for affinity with the Aphyllophorales. Corner [31, 32] brings Cantharellus in with the large simple Clavaria Clavariadelphus: Cl. pistillaris possesses the veined hymenium of the Chanterelles, Cl. truncatus has a truncated and sterile top which cannot be very far removed from the origin or regression of a pileus. According to this author, apart from the arrangement of the basidium divisions - to which character he accords little weight - the two genera differ only by the presence of a true pileus in Cantharellus; but Clavariadelphus truncatus acquires marginal growth and the fructification considerably ressembles that of Canth. culticulatus; conversely, if Cantharellus lost its marginal growth, the result would be Cl. truncatus. The direction in which this evolution, if such it is, took place remains to be defined. Donk [40], however, assigning great importance to the chiastobasidial or stichobasidial character of the basidia, makes a sharp distinction between Cantharellus and Clavariadelphus, since in his opinion the morphological similarity of the two genera is a mere convergence phenomenon, and would rather relate the latter to Gomphus. The views of Petersen are closer to those of Corner as to the importance of the arrangement of the mitoses in the basidia [124], and his study of spore staining by cotton blue [125] flatly contradicts the opinion of Donk, clearly separating Clavariadelphus from Gomphus and placing them with Cantharellus in a vast collection which also includes the Clavaria-Clavulinopsis series. /70

It appears to us that Cantharellus, rich in carotenoids, cannot be derived from Clavariadelphus which has no such pigments: if indeed a relationship exists between these two genera, evolution would have proceded from a still-chiastobasidial pre-Cantharellus (if, with Corner [31], Boidin [18], Petersen [124] we consider the stichobasidial mode of division to have appeared second) to Clavariadelphus. On the other hand, the identity of pigmentation, the macroscopic and microscopic elements described by Kühner, and the odor together comprise a sheaf of characters

common to Omphalia chrysophylla and the Chanterelles and appearing in no other group. This leads us to think that the origin of the Chanterelles must be sought around this Omphalia, if we consider the stichobasidial character as evolved. In any case, it seems going too far to exclude the Chanterelles from the Agaricales. The true place in this order of many cantharelloid species has been recognized; the same thing seems to go for the Chanterelles proper (Cantharellus) which seems to us to be an offshoot whose origin is probably very close to the Gerronema genus two species of which, as already shown, are carotenogenous and often have the yellow spores of many Chanterelles.

3. CONCLUSIONS

Although this study has provided material for but few firm conclusions, this very fact is encouraging since it shows how much remains to be done in this field. We can expect fertile data to be forthcoming on the properties and thence the interrelationships of many a system group. The cantharelloid fungi, often carotenogenous, take pride of place here, but also the Agaricales which may be close, as they synthesize the same pigments, and also the clavarioid fungi. The relationship that seems to be there between the pigmentation of Pistillaria micans and Clavaria helicoïdes raises the question of possible relations between these two species and, correlatively, the genera to which they belong, or the question of the position of Cl. helicoides with respect to the other red Clavaria (is this color due to carotenoids?) /71
There is also the question of the foreign Clavaria with carotenoids: cantharelloids according to Heim [14]. Better knowledge of their pigments, and generally of the distribution of carotenoids in Clavaria in the broadest sense of the term, would doubtless bring forth interesting arguments in the discussion of the relations inside the group and those with cantharelloid fungi.

This brings us to the need for the mycologist (in particular) not to restrict himself to European flora alone, but to give weight to exotic species: Cl. helicoïdes and Cl. cardinalis, for instance. In the taxonomic study of Discomycete carotenoids, analysis of Central African Republic species has proved particularly fruitful [10, 11]. The fact is evidently one of quite general application: we are familiar with the classical example of the European "ancestors" of the horse, and man himself must seek his origins in Africa or the Far East.

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